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TITLE OF THESIS:
DEVELOPMENT OF BIOLOGIC DEVICES FOR MESOTHELIN
IMMUNOTARGETING

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Contents

ABBREVIATIONS	7
<hr/> RIASSUNTO	<hr/> 9
<hr/> ABSTRACT	<hr/> 11
<hr/> INTRODUCTION	<hr/> 13
<hr/> THE CANCER PROBLEM FROM AN IMMUNE SYSTEM PERSPECTIVE	<hr/> 13
<hr/> <i>Active Cancer Immunotherapy:</i>	<hr/> 15
<hr/> <i>Passive Cancer Immunotherapy:</i>	<hr/> 15
<hr/> MONOCLONAL ANTIBODIES: VERSATILE PLATFORMS FOR CANCER IMMUNOTHERAPY AND DIAGNOSTIC APPLICATIONS	<hr/> 17
<hr/> <i>Murine monoclonal antibodies:</i>	<hr/> 19
<hr/> <i>Chimeric and humanized monoclonal antibodies:</i>	<hr/> 19
<hr/> <i>Fully human monoclonal antibodies:</i>	<hr/> 20
<hr/> <i>Recombinant antibody constructs:</i>	<hr/> 20
<hr/> MECHANISM OF ACTION OF THERAPEUTIC MONOCLONAL ANTIBODIES	<hr/> 21
<hr/> <i>Immune system activation:</i>	<hr/> 21
<hr/> <i>Blockade of ligand binding and signaling perturbation:</i>	<hr/> 24
<hr/> <i>Conjugates:</i>	<hr/> 25
<hr/> DIAGNOSTIC APPLICATION OF ANTIBODIES	<hr/> 28

<i>Radioimmunosintigraphy</i>	30
<i>Immuno-PET</i>	32
MESOTHELIN EXPRESSION IN HUMAN CANCERS	35
<i>Mesothelioma</i>	36
<i>Pancreatic cancer</i>	37
<i>Ovarian cancer</i>	38
<i>Lung cancer</i>	38
<i>Mesothelin expression in other human tumors</i>	39
MESOTHELIN BIOLOGY	40
<i>Mesothelin binding to MUC16/CA-125 and ovarian cancer</i>	40
<i>Mesothelin regulation by Wnt-1 and Wnt-5a</i>	41
<i>Humoral anti-mesothelin immune response in cancer</i>	42
<i>Prognostic significance of mesothelin expression in ovarian cancer</i>	42
MESOTHELIN TARGETED THERAPIES	43
<i>SSIP (CAT-5001)</i>	43
<i>MORAb-009</i>	45
<i>CRS-207</i>	45
<i>Mesothelin cancer vaccines</i>	46
<i>Mesothelin targeted ovarian cancer gene therapy</i>	47

<i>Mesothelin as a target for radioimmunotherapy</i>	47
--	----

IMMUNOTOXIN DEVELOPMENT	50
--------------------------------	-----------

RIBOSOME INACTIVATING PROTEINS BASED IMMUNOTOXINS	53
--	-----------

<i>Distribution in nature and mechanism of action</i>	54
---	----

<i>Toxicity</i>	56
-----------------	----

<i>Immunotoxins and other conjugates</i>	57
--	----

AIM OF THE THESIS	59
--------------------------	-----------

MATERIAL AND METHODS	61
-----------------------------	-----------

<i>PLASMID pcDNA3.1 STRUCTURE</i>	61
-----------------------------------	----

<i>CELL LINES</i>	62
-------------------	----

<i>PRODUCTION OF RECOMBINANT MESOTHELIN</i>	63
---	----

<i>TRANSFECTION</i>	65
---------------------	----

<i>BALB/c MICE IMMUNIZATION FOR ANTI-MESOTHELIN ANTIBODY</i>	
--	--

<i>PRODUCTION</i>	66
-------------------	----

<i>ADJUVANTS</i>	67
------------------	----

<i>PREPARING MACROPHAGE FEEDER CELL CULTURES</i>	67
--	----

<i>PREPARING SPLENOCYTES FOR FUSION</i>	68
---	----

<i>PREPARING MYELOMA CELLS FOR FUSION</i>	68
---	----

<i>FUSION PROTOCOL</i>	69
<i>FACS (Fluorescence Activated Cell Sorter)</i>	72
<i>mRNA EXTRACTION</i>	73
<i>RETROTRANSCRIPTION AND cDNA SYNTHESIS</i>	74
<i>POLYMERASE CHAIN REACTION (PCR)</i>	76
<i>AGAROSE GEL ELECTROPHORESIS</i>	77
<i>MONOCLONAL ANTIBODIES PURIFICATION</i>	78
<i>SDS PAGE (Sodium - Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis)</i>	79
<i>BLUE COMASSIE STAINING</i>	80
<i>BCA (bicinchoninic acid assay)</i>	80
<i>mAb ANTI-MESOTHELIN/RTA CHEMICAL IMMUNOTOXIN</i>	81
<i>XTT cell viability assay</i>	81
RESULTS	85
<i>TOTAL RNA EXTRACTION FROM AN OVARIAN CANCER TISSUE</i>	85
<i>RT-PCR END PCR AMPLIFICATION OF MESOTHELIN-SPECIFIC cDNA</i>	85
<i>RECOMBINANT MESOTHELIN PRODUCTION</i>	86
<i>MESOTHELIN TRANSFECTED HUMAN HEK293 CELLS</i>	88

BALB/c IMMUNIZATION WITH MESOTHELIN EXPRESSING CELL MEMBRANES 91

<i>GENERATION AND GROWTH OF HYBRIDOMAS</i>	<i>93</i>
--	-----------

<i>FROM POLYCLONAL TO MONOCLONAL HYBRIDOMA</i>	<i>95</i>
--	-----------

<i>GROWTH OF THE CLONES IN HYBRIDOMED MEDIUM</i>	<i>100</i>
--	------------

<i>PURIFICATION AND CHARACTERIZATION OF THE NEW mAbs</i>	<i>101</i>
--	------------

<i>SYNTHESIS OF THE ANTI MESOTHELIN IT: mAb –spdp-nRTA</i>	<i>107</i>
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<i>“IN VITRO” IMMUNOTOXIN EFFICACY</i>	<i>108</i>
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<i>DISCUSSION</i>	<i>113</i>
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<i>REFERENCES</i>	<i>123</i>
-------------------	------------

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
BSA	Bovine Serum Albumin
Da	Dalton
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovin Serum
FITC	Fluorescein Isothiocyanate
IB	Inclusion Bodies
Ig	Immunoglobuline
IHC	Immunohistochemistry
IT	Immunotoxin
mAb	Monoclonal Antibody

MFI	Mean Fluorescence Intensity
MW	Molecular Weight
PBS	Phosphate Buffered Saline
PE	Pseudomonas Aeruginosa Exotoxin A
RIP	Ribosome inactivating protein
RTA	Ricin A chain
scFv	Single Chain Variable Fragment
SPDP	N-Succinimidyl-3-(2 - Pyridylthio) Propionate
WB	Western Blot
Wt	Wild Type
³HTdr	Triziate – Thymidine

RIASSUNTO

La mesotelina è una glicoproteina di superficie cellulare sintetizzata a partire da un precursore di 71 kDa che viene processato in un frammento di 40 kDa legato alla membrana ed un fattore solubile potenziante i megacariociti di 31 KDa. La mesotelina rappresenta un promettente biomarcatore tumorale; in condizioni fisiologiche è espressa dalle cellule mesoteliali delle sierose dell'organismo (pleura, pericardio e peritoneo) mentre è overespressa sulla superficie cellulare di diversi istotipi tumorali tra cui: il mesotelioma maligno, il carcinoma ovarico, pancreatico e polmonare. La sua funzione è sconosciuta ma recentemente è stato ipotizzato un suo ruolo nei meccanismi di adesione cellulare attraverso il legame all'antigene tumorale CA125.

La limitata espressione della mesotelina a livello dei tessuti fisiologici e, di contro, l'elevata espressione in molti tumori, rendono questo antigene un possibile target nella immunoterapia anti-tumorale. Il presente lavoro descrive la generazione di un nuovo anticorpo monoclonale anti-mesotelina ottenuto mediante la tecnologia degli ibridomi e la caratterizzazione delle sue proprietà di riconoscimento. Tale anticorpo monoclonale è stato testato per valutare un suo potenziale utilizzo terapeutico e diagnostico.

L'anticorpo anti-mesotelina ha dimostrato, in analisi condotte mediante citofluorimetria, una notevole specificità, essendo in grado di riconoscere cellule che esprimono l'antigene mesotelina costitutivamente (OVCAR-3) e per trasfezione (HEK293-mesotelina); allo stesso tempo l'anticorpo ottenuto non possiede la capacità di legare

cellule mesotelina negative. La specificità di riconoscimento in vitro è paragonabile all'anticorpo monoclonale commerciale K1 da noi utilizzato come controllo positivo.

Studi di binding, inoltre, hanno permesso di evidenziare la migliore affinità del nostro anticorpo rispetto all'anticorpo monoclonale commerciale K1.

Basandoci su questi dati abbiamo sviluppato una immunotossina chimica con la capacità di riconoscimento del nostro anticorpo e l'attività citotossica della ricina (una potente tossina appartenente alla famiglia delle proteine inattivanti i ribosomi) con lo scopo di poter utilizzare tale costrutto nella immunoterapia passiva di tumori overesprimenti la mesotelina. Le frazioni raccolte e purificate dell'immunotossina, ottenute mediante la produzione di un ponte disolfuro tra l'anticorpo monoclonale e la ricina, risultano capaci di inibire del 50% la proliferazione cellulare (IC_{50}). Tale attività citotossica si manifesta su cellule mesotelina positive ad una concentrazione di 0,03 nM e 0,09 nM rispettivamente a 36 h e 72 h di incubazione. Inoltre il nostro costrutto dimostra una elevata specificità di riconoscimento in quanto non sono stati raggiunti valori di IC_{50} su cellule mesotelina-negative anche dopo aggiunta di concentrazioni pari o superiori a 78 nM dell'immunotossina. Si è infine osservato che l'attività citotossica di una concentrazione pari a 0,03 nM dell'immunotossina è completamente neutralizzata dalla contemporanea aggiunta, nel terreno di coltura, dell' anticorpo anti-mesotelina da noi generato ad una concentrazione 1000 volte maggiore rispetto a quella dell'immunotossina. Questo ultimo dato supporta i precedenti ottenuti potenziando l'evidenza di una attività citotossica specifica e anticorpo mediata da parte della nostra immunotossina chimica.

ABSTRACT

BACKGROUND: Mesothelin is a tumor differentiation antigen (Ag) that is normally present on the mesothelial cells lining of the pleura, peritoneum and pericardium. It is, however, highly expressed in several human cancers including malignant mesothelioma, pancreatic, ovarian and lung adenocarcinoma. The normal biologic function of mesothelin is unknown but recent studies have shown that it binds to CA-125 and may play a role in the peritoneal spread of ovarian cancer. The limited mesothelin expression in normal tissues and high expression in many cancers makes it an attractive candidate for cancer immunotherapy.

RESULTS: In this study we have developed a monoclonal antibody (mAb) that is specific for the Ag mesothelin. It was produced by hybridomas technologies and we have performed Fluorescence Activated Cell Sorting (FACS) analysis to evaluate the specificity and affinity of this antibody for the Ag of interest. The mAb binds mesothelin-positive cell lines expressing the Ag constitutively (OVCAR-3) and transfected cells (HEK293-mesothelin). The same mAb not recognizes mesothelin-negative cell lines demonstrating an high specificity in vitro. Binding studies have demonstrated that our mAb has a better affinity with respect to mAb K1, a commercially available anti-mesothelin mAb.

Our anti-mesothelin mAb was chemically linked to ricin A chain (RTA) toxin obtaining a powerful immunodelivered drug (immunotoxin, IT) with specific cytotoxic activity on mesothelin positive cells; in a cytotoxic assay on HEK293-mesothelin transfected cells

the anti-mesothelin mAb-RTA IT shows an IC_{50} of 0.03 nM and 0,09 nM after 36 hrs and 72 hrs of incubation respectively; no cytotoxic activity was observed against mock-not transfected one and other mesothelin negative cells. As a further proof of specificity we observed that the cytotoxic activity of 0,03 nM of the above-mentionated IT on HEK293-mesothelin cells, is fully prevented by addition of whole molecule mesothelin-specific antibody at a concentration 1000 fold over IT.

CONCLUSION: Our mAb holds great potential to be used as a research reagent and diagnostic tool in research laboratories and in the clinics because of its high quality and versatility. This antibody is also a strong candidate to be investigated for further in vivo passive immunotherapy studies. Moreover, the discovered of this new mAb anti-mesothelin enable us to develop in vivo diagnostic approaches using radionuclide, fluorescence trackers or nanoparticles. Its conjugation with therapeutic molecules allows a better distribution of the drug to the tumor sites; this ability increase the antitumor efficacy and reduce the specific toxicity at the same time.

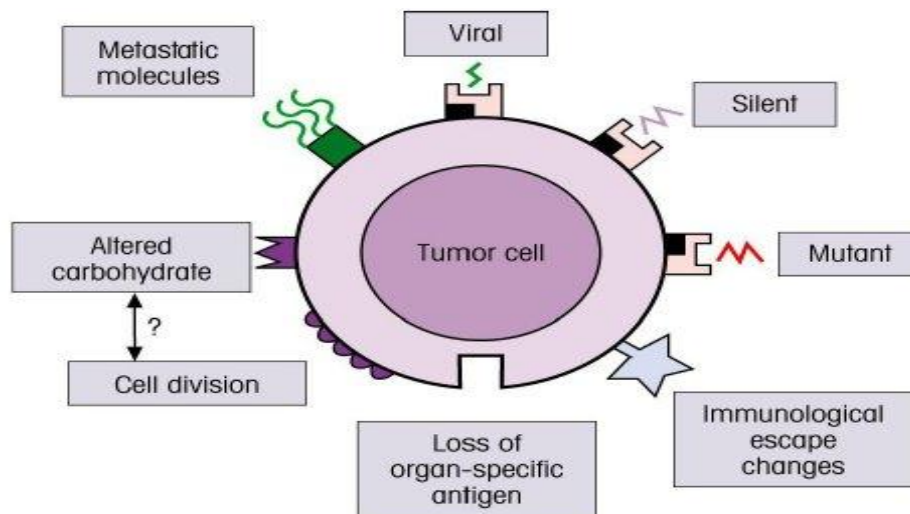
INTRODUCTION

Cancer malignancies are among the most life-threatening diseases in industrialized countries. Although established cancer therapies, including surgical resection of the primary tumor, radiation and chemotherapy, are well established, cancer still causes 25% of mortalities. Annually, about 1% of the population that has been diagnosed with cancer, die. Five-year survival rates range from 10-20% for lung, esophagus and stomach cancer, to 40-60% for colon, bladder and cervix cancer, and 60-80% for breast and prostate cancer (www.rex.nci.nih.gov). Whereas the primary tumor can in most cases be treated efficiently by a combination of standard therapies, preventing the metastatic spread of the disease through disseminated tumor cells is currently ineffective.

THE CANCER PROBLEM FROM AN IMMUNE SYSTEM PERSPECTIVE

Cancer immunotherapy – as an alternative modality of treatment – has significantly developed during the last decades and completes the therapeutic arsenal. In contrast to the other therapeutic concepts, immunotherapy primarily aims to prevent metastatic spread of the disease and to improve quality of life of the affected individuals. Approaches that are applied in immunotherapy are based on complementation or stimulation of the immune system via a plethora of compounds, such as lymphokines, vaccines, in vitro-stimulated effector cells of the immune system or antibodies. Immune surveillance is the concept that envisages prevention of the development of tumors by

the early destruction of abnormal cells by the immune system of the host. Consequently, a lack of (or escape from) immune surveillance plays an essential role in cancer development, and may be associated with an evasion of tumor cells from the surveillance of the immune system, which in particular seems to be an escape from specific T-cell mediated immunity [1-2]. Due to their genetic instability, tumor cells usually express abnormal proteins, *i.e.*, tumor-associated antigens (TAAs), which have no or very limited expression on normal cells [3-4]. Such TAAs expose new, potentially immunogenic epitopes, which can be recognized by the immune system of the host (Figure 1).



Delves et al. Roitt's Essential Immunology, 12th ed.
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Fig. 1 Tumor-associated surface changes.

Interestingly, the endogenous immune response against these epitopes has only a marginal effect on the tumor [5]. With progression of tumor growth, the cytotoxic

Introduction – Cancer Immunotherapy

immune response at the tumor site has been found to be inhibited, and invading T cells and antigen-presenting cells (APCs) often appear to be non-functional [6-7]. Moreover, tumor themselves have been shown to down-regulate the immune surveillance by secreting immunosuppressant factors like interleukin IL-10 or transforming growth factor- β [8-9].

Active Cancer Immunotherapy:

cancer immunotherapy is intended to restore reactivity of the host's immune system to combat cancer. Active cancer immunotherapy aims at induction of an endogenous, long-lasting tumor antigen-specific immune (preventive or therapeutic) response [10] (Figure 2). For this specific vaccination approach defined TAAs or material obtained after biopsy of the tumor is used [3-4]. The immune response against the tumor can be further enhanced by unspecific stimulation of the immune system using stimulators such as adjuvants or so-called biological response modifiers (*e.g.* cytokines). An alternative to the induction of an immune response *in vivo* (by administration of a tumor antigen as a vaccine to the host's APCs) is *ex vivo* stimulation of autologous dendritic cells (DCs) with re-application of the DCs to the patient, as a method to break the patient's immune tolerance for TAAs.

Passive Cancer Immunotherapy:

passive cancer immunotherapy, on the other hand, provides a tumor antigen-specific immune response by supplying high amounts of effector molecules, *i.e.*, tumor-specific antibodies or effector cells, *i.e.*, cytotoxic T lymphocytes. In contrast to active

immunization, passive cancer immunotherapy is short lived, and dependent on repeated applications [10]. Antibodies as part of the humoral immune system recognize antigenic structures, neutralize them or mediate effector functions like antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) (Figure 2). Cancer immunotherapy should take into account the genetic instability of the tumor cells, as they rapidly adapt to changes in their environment. Monovalent therapies targeting only one epitope may, therefore, not provide long-term protection because tumor cells may escape immune surveillance by down-regulating the targeted antigen [1]. Multi-epitope targeting approaches could strongly reduce evasive cancer potential, and may eventually prevent tumor escape [11].

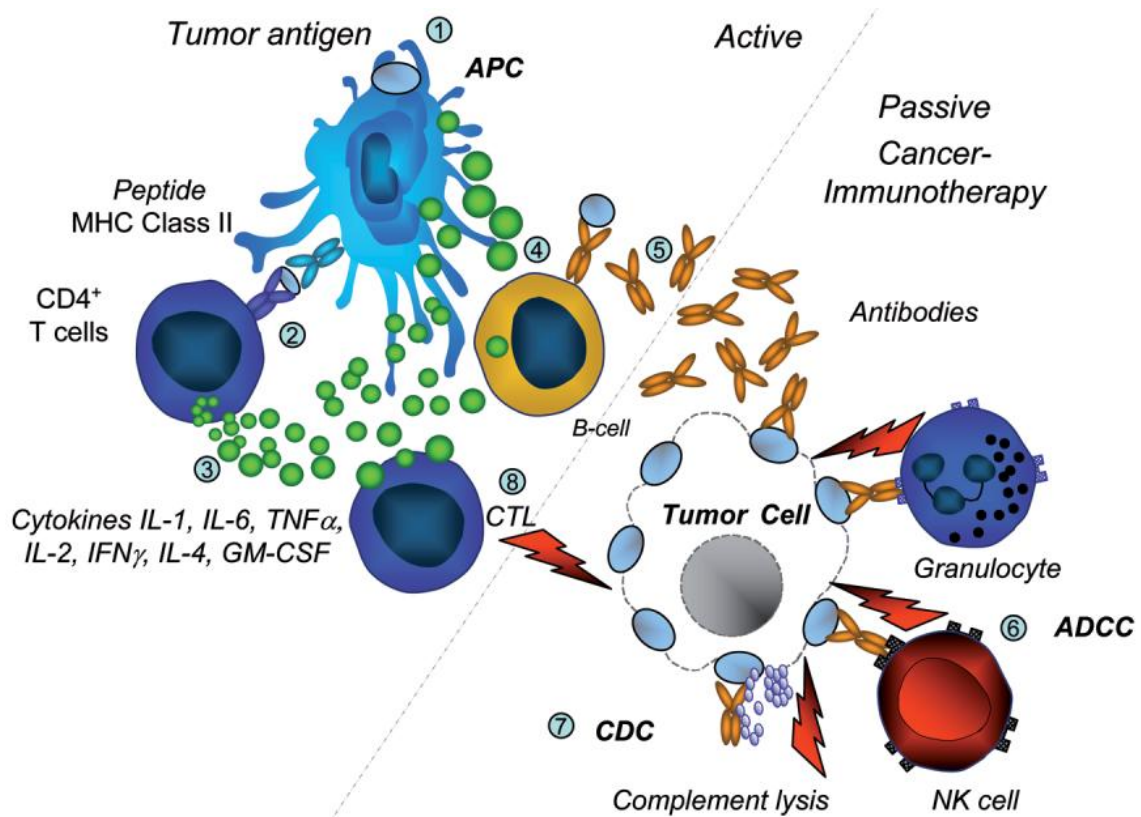


Fig. 2 Cancer Immunotherapy. The whole spectrum of passive and active cancer immunotherapy is summarized. Active cancer immunotherapy comprises tumor antigen uptake by APCs (1), epitope (peptide) presentation to CD4⁺ T cells (2), cytokine release (3), B cell activation (4), and antibody production (5), leading to lysis of tumor cells including different (passive) alternatives like ADCC (6), CDC (7) or unspecific attack by cytotoxic T lymphocytes (8) (Schuster M., *et al.*, 2006).

MONOCLONAL ANTIBODIES: VERSATILE PLATFORMS FOR CANCER IMMUNOTHERAPY AND DIAGNOSTIC APPLICATIONS

Antibodies are grouped into five classes based on the sequence of their heavy chain constant regions: IgM, IgD, IgG, IgE and IgA. Of the five classes, IgG is the most frequently used for cancer immunotherapy. Antibodies can be subdivided into two distinct functional units: the fragment of antigen binding (Fab) and the constant fragment (Fc). The Fab contains the variable region, which consists of three

hypervariable complementarity-determining regions (CDRs) that form the antigen binding site of the antibody and confer antigen specificity. Antibodies are linked to immune effector functions by the Fc fragment, which is capable of initiating CDC, binding to Fc receptors for IgG (FcγRs) and binding to the neonatal FcR (FcRn) [12].

The concept of using antibodies to selectively target tumors was proposed by Paul Ehrlich over a century ago [13]. The advent of hybridoma technology in 1975 enabled the production of monoclonal antibodies [14]. Owing to their origins in mice, these monoclonal antibodies were typically immunogenic in humans and had poor abilities to induce human immune effector responses, thereby limiting their clinical applicability. Later advances in antibody engineering provided flexible platforms for the development of chimeric, humanized and fully human monoclonal antibodies which satisfactorily addressed many of these problems (see Timeline).

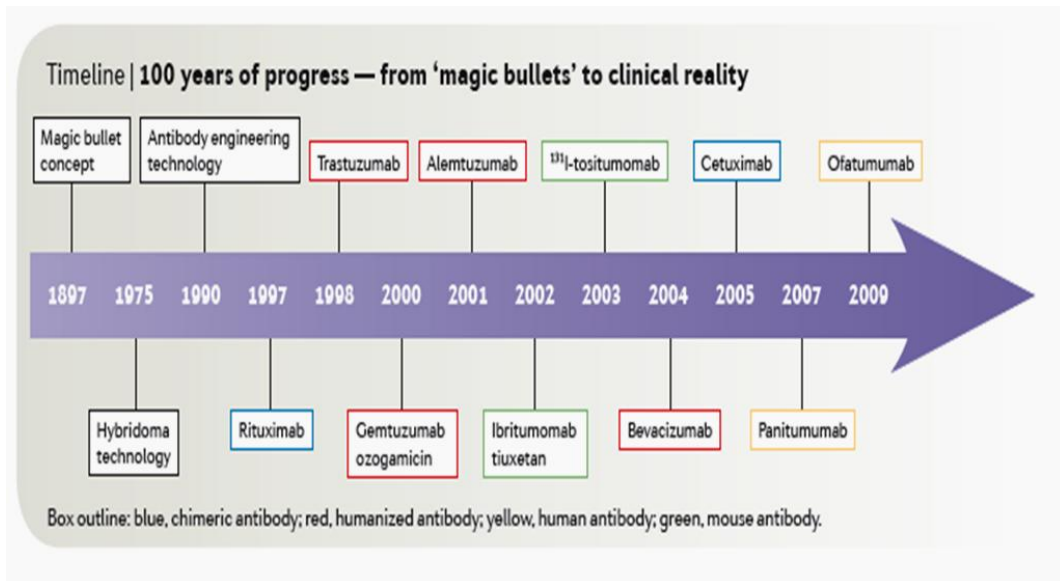


Fig. 3 TIMELINE Box outline: blue, chimeric antibody; red, humanized antibody; yellow, human antibody; green, mouse antibody (Louis M. Weiner, *et al.*, 2010).

Murine monoclonal antibodies:

these antibodies are derived entirely from mice using hybridoma technology, which involves the fusion of immortalized myeloma cells with B-cells from immunized mice. In humans, these antibodies often had limited clinical utility because they have short circulating half-lives [15], are immunogenic [16-17] and have difficulties inducing human immune effector responses [18]. Their main use has been to serve as targeting agents for radioisotopes or cytotoxins that kill targeted tumor cells. To date, two radiolabelled murine antibodies, *ibritumomab* tiuxetan and *tositumomab*, have been approved for use in cancer therapy (Table 1) [19].

Chimeric and humanized monoclonal antibodies:

the desire to reduce the immunogenicity and to increase the immunologic efficiency of antibodies led to the production of chimeric and humanized mAbs [20–21]. Chimeric mAbs are constructed by fusing the murine variable regions onto human constant regions. Humanized mAbs were constructed by grafting murine antigen binding regions (CDRs) onto human antibodies. Unfortunately, humanized mAbs often have considerably poorer affinity than the parent murine mAb and require further manipulation (typically by introducing mutations in the CDRs) to restore the affinity and specificity of the original murine mAb [22]. The majority of approved anticancer mAbs are humanized antibodies (Table 1).

Fully human monoclonal antibodies:

the immunogenicity of therapeutic mAbs affects their efficacy and safety. Patient immune responses raised against the therapeutic mAbs can neutralize their action, whereas hypersensitivity to these mAbs can result in morbidity and mortality. Fully human mAbs were developed to further reduce immunogenicity associated with chimeric or humanized mAbs, both of which still retain some murine components [23-24]. Human mAbs have been produced using either transgenic mice (transgenic animals bearing a ‘human’ immune system) or phage display technology [25]. Elimination of the murine protein sequences has generally been found to reduce the frequency of mAb-targeted immune responses and hypersensitivity reactions [26]. *Panitumumab* and *ofatumumab* are two human mAbs that have been approved for use in the clinic (Table 1). Both these mAbs were produced by transgenic mice technology. *Panitumumab* was produced with the Abgenix Xeno-Mouse technology and *ofatumumab* was generated from the UltiMab platform [23].

Recombinant antibody constructs:

several recombinant antibody constructs have been produced to increase affinity, improve penetration and retention by solid tumors, mediate enhanced human immune effector responses and demonstrate favourable plasma pharmacokinetics. The targeting specificity of whole mAbs can be retained in these recombinant constructs. Another advantage is that these constructs can often be produced at lower cost [27]. Examples of such constructs include single chain-variable fragments (scFvs), multivalent scFvs (e.g.

diabodies and tribodies), minibodies (e.g. scFv-CH3 dimers), bispecific antibodies, Fab and Fab₂ fragments and camel variable functional heavy chain domains (see reviews by Beckman et al. [28] and Hudson [29]). No such construct has yet been approved for use in humans.

MECHANISM OF ACTION OF THERAPEUTIC MONOCLONAL ANTIBODIES

Immune system activation:

antibodies are thought to function through several different mechanisms, although antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are thought to be particularly important. ADCC involves destruction of the antibody-coated cell by recruitment of effector cells (such as natural killer cells, macrophages and neutrophils) whereas CDC involves destruction through complement activation. Both ADCC and CDC are mediated via the Fc region of the antibody, with mAbs of the IgG1 isotype being most effective at inducing ADCC and CDC [12-19-30-31]. FDA approved mAbs that are believed to use ADCC as their primary mode of action are *rituximab* and *ofatumumab*; however their activity is also thought to partially depend on CDC. By contrast, *alemtuzumab* induces CDC but not ADCC [17]. Certain mAbs that primarily function by disrupting signalling pathways have also been shown to induce ADCC (Table 1). ADCC occurs when antibodies bind to antigens on tumor cells and the Fc portion of the mAb engages Fc γ receptors (Fc γ Rs) on the surface of immune effector cells. A seminal paper by Ravetch and colleagues (*Nature*

Immunology, 2010) demonstrated the importance of Fc-Fc γ R interactions for the in vivo antitumor effects of *trastuzumab* and *rituximab*. This study showed that the antitumor activities of these mAbs were lower in Fc γ R-deficient mice than in wild-type mice [25]. The importance of the Fc γ R in the antitumor response of therapeutic mAbs has been further supported by the discovery that polymorphisms in the genes encoding Fc γ Rs, that lead to a higher affinity of mAbs for the Fc γ R, are associated with higher clinical response rates in patients. Polymorphisms in the Fc γ R genes have been shown to be associated with clinical responses to *rituximab*, *trastuzumab* and *cetuximab* [32-33-34]. The ability of therapeutic mAbs to mediate ADCC is also influenced by its oligosaccharide content. The majority of currently used mAbs are highly fucosylated because of the nature of the cell lines used for their production. Generation of mAbs with defucosylated oligosaccharides has been shown to enhance ADCC in vitro and enhance antitumor activity in vivo [17-23]. Tumors can escape the immune system by inducing regulatory T cells which function to suppress the patient's immune responses. Specific antigens expressed on the surface of these regulatory T cells which are involved in immunosuppression, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), are therefore attractive targets for therapeutic mAbs. Such immunomodulation might allow for a more effective immune response to the tumor. *Ipilimumab* and *tremelimumab* are two anti-CTLA-4 blocking mAbs that have been studied in clinical trials and shown to induce delayed disease regression in patients with metastatic melanoma [24].

TABLE 1
Monoclonal antibodies currently FDA approved for clinical use and mechanisms of action

Antibody	Target	FDA approved indication (September 2010)	Comments regarding usage in Europe and Australia ^b	Mechanisms of action
Naked antibodies – solid malignancies				
Trastuzumab (Herceptin [®]) – humanised IgG1	HER2 (ErbB2)	HER2-positive breast cancer – as single agent or in combination with chemotherapy for (i) adjuvant or (ii) palliative treatment HER2-positive gastric or gastroesophageal junction carcinoma – as first-line treatment in combination with cisplatin and capecitabine/S-FU	Similar	Inhibition of HER2 signalling; ADCC
Bevacizumab (Avastin [®]) – humanised IgG1	VEGF	For the palliative treatment of colorectal cancer, non-squamous non-small cell lung cancer, HER2-negative breast cancer, glioblastoma or renal cell carcinoma	Similar	Inhibition of VEGF signalling
Cetuximab (Erbix [®]) ^a – chimeric human/murine IgG1	EGFR (ErbB1)	In combination with radiation therapy for the initial treatment of locally or regionally advanced SCCHN; as a single agent for SCCHN patients with whom prior platinum-based therapy has failed; palliative treatment of pre-treated metastatic EGFR-positive colorectal cancer	Similar	Inhibition of EGFR signalling; ADCC
Panitumumab (Vectibix [®]) ^a – human IgG2	EGFR (ErbB1)	As a single agent for the treatment of pre-treated EGFR-expressing, metastatic colorectal carcinoma	Similar	Inhibition of EGFR signalling
Naked antibodies – haematological malignancies				
Rituximab (Mabthera [®]) – chimeric human/murine IgG1	CD20	For the treatment of CD20 positive B-cell NHL and CLL	Similar	ADCC; direct induction of apoptosis; CDC
Alemtuzumab (Campath [®]) – humanised IgG1	CD52	As a single agent for the treatment of B-cell CLL	Similar	Direct induction of apoptosis; CDC
Ofatumumab (Arzerra [®]) – human IgG1	CD20	Treatment of patients with CLL refractory to fludarabine and alemtuzumab	Similar in Europe but not approved in Australia	ADCC; CDC
Conjugated antibodies – haematological malignancies				
Gemtuzumab ozogamicin (Mylotarg [®]) – humanised IgG4	CD33	For the treatment of patients with CD33 positive acute myeloid leukaemia in first relapse who are 60 years of age or older and who are not considered candidates for other cytotoxic chemotherapy	Not approved in the European Union or Australia	Delivery of toxic payload, calicheamicin toxin
⁹⁰ Y-ibritumomab Tiuxetan (Zevalin [®]) – murine IgG1	CD20	Treatment of relapsed or refractory, low grade or follicular B-cell NHL; previously untreated follicular NHL in patients who achieve a partial or complete response to first-line chemotherapy	Similar in Europe but not approved in Australia	Delivery of the radio isotope yttrium-90
¹³¹ I-Tositumomab (Bexxar [®]) – murine IgG2	CD20	Treatment of patients with CD20 antigen expressing relapsed or refractory, low grade, follicular, or transformed NHL	Granted orphan status drug in 2003 in European Union but not approved in Australia	Delivery of the radio isotope iodine-131; ADCC; direct induction of apoptosis

ADCC, antibody-dependent cell cytotoxicity; CDC, complement-dependent cytotoxicity; CLL, chronic lymphocytic leukaemia; EGFR, epidermal growth factor receptor; NHL Non-Hodgkin's Lymphoma; SCCHN, squamous cell carcinoma of the head and neck; VEGF, vascular endothelial growth factor.

^a Not recommended in colorectal cancer patients whose tumours expressed mutated K-ras.

^b Based on information from the European Medicines Agency and the Australian Therapeutic Goods Association.

Table 1 Since 1997, ten mAbs have received approval from the United States Food and Drug Administration (US FDA) for the treatment of a variety of solid tumours and haematological malignancies, and a large number of additional therapeutic antibodies are currently being tested in early and late stage clinical trials (<http://www.clinicaltrials.gov>) (Vinochani Pillay *et al.*, 2011).

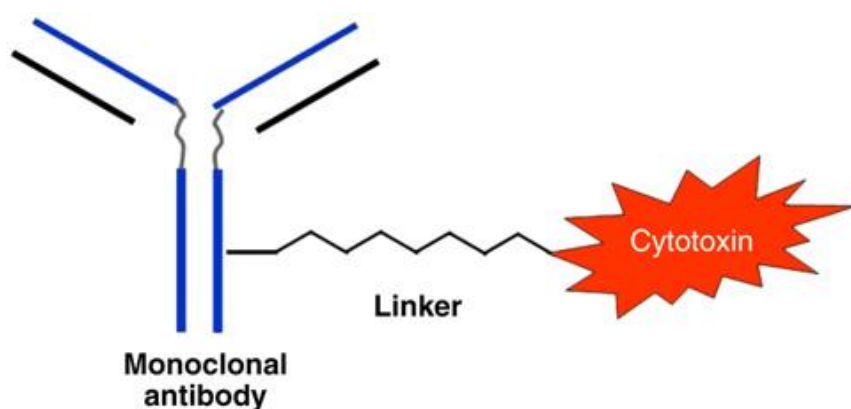
Blockade of ligand binding and signaling perturbation:

Increased expression of growth factor receptors during tumorigenesis also make them attractive targets for therapeutic antibodies (Table 1). mAbs can exert antitumor effects by blocking ligand binding to growth factor receptors and inhibiting signaling through these receptors, thereby inhibiting proliferation of the targeted cells, inducing apoptosis and/or sensitizing tumors to chemotherapeutic agents [19-32]. Two approved mAbs, *cetuximab* and *panitumumab*, are believed to function primarily by binding to and blocking epidermal growth factor receptor (EGFR, also called ErbB1) mediated signaling. Because EGFR enhances both tumor cell proliferation and neoangiogenesis, inhibition of this signalling pathway reduces cell proliferation and tumor angiogenesis. Inhibition of EGFR signalling also sensitizes the cells to chemotherapy and radiation [35]. HER2 (or ErbB2) belongs to the same family as EGFR but has a truncated extracellular domain that is unable to bind ligand. Antibodies targeting this receptor function mainly by inhibiting receptor homo and hetero-dimerization and internalization rather than preventing ligand binding [36-37]. *Trastuzumab*, which is used for the treatment of metastatic breast cancer, selectively inhibits HER2 homodimer-induced tyrosine kinase activation, whereas *pertuzumab* blocks HER2 dimerization with any ErbB receptor [38]. An approved antibody, *bevacizumab*, blocks binding of vascular endothelial growth factor (VEGF) to its receptor and inhibits tumor angiogenesis [39–40]. Two other growth factor receptors that have received some interest as potential targets for mAb therapy are the receptor tyrosine kinases c-MET and insulin-like growth

factor receptor 1 (IGFR1). mAbs targeting c-MET and IGFR1 are currently under clinical development (<http://www.clinicaltrials.gov>).

Conjugates:

despite these advances, there are still limitations to mAb therapeutics, including poor penetration into tumors, with only a small percentage of the administered mAb localizing within the tumor, and slow extravasation of the drug from the blood supply [41-42]. Additionally, antibodies are expensive to generate, which increases the costs of the marketed drugs [43-44]. Monoclonal antibody therapies are often not curative and may need to be combined with chemotherapy [45]. As a result, there has been tremendous interest in finding a way to increase the therapeutic effectiveness of the mAb through attachment to a toxin, drugs, or radionuclides. Antibody drug conjugates (ADCs) consist of an antibody attached to a cytotoxic drug by means of a linker (Figure 4). ADCs provide a way to couple the specificity of a monoclonal antibody (mAb) to the cytotoxicity of a small molecule drug and, therefore, are promising new therapies for cancer. ADCs are prodrugs that are inactive in circulation but exert their cytotoxicity upon binding to the target cancer cell.



mAb	Linker	Drug	Name of ADC
Trastuzumab	Disulfide	Maytansine	Trastuzumab – DM1
Anti-CD22	Hydrazone	Calicheamycin	Inotuzumab ozogamicin
Anti-CD30	Peptide	Auristatin	SGN-35

Fig. 4 Schematic of an ADC. The schematic for the structure of an ADC is shown along with examples of antibodies, linkers and drugs used to make some of the ADCs currently in clinical trials (Iyer U., *et al.*, 2011).

Yttrium-90 (Y-90) and iodine-131 (I-131) are two of the most common isotopes conjugated to mAbs in the clinic [19]. Two radiolabelled murine mAbs, namely Y-90 *ibritumomab* tiuxetan and I-131 *tositumomab*, have been approved for Non-Hodgkin's Lymphoma (NHL, Table 1). Calicheamicin is a highly potent toxin that binds to DNA, causing double-strand breaks and cell death by apoptosis. *Gemtuzumab* ozogamicin is a calicheamicin immunoconjugate targeted to CD33 that has been approved for the treatment of acute myeloid leukaemia (AML) (Table 1). Examples of other calicheamicin conjugates that have been evaluated in the clinic include CMB-401 and

CMD-193, where the calicheamicin is conjugated to the anti-MUC1 antibody CTM01 [46] or the anti-Lewis Y antibody hu3S193 [47-48], respectively. Other toxins that have received recent attention for development of immunoconjugates include maytansinoids, ricin A and Pseudomonas exotoxin A [35]. Maytansinoids are a naturally occurring group of highly potent cytotoxic agents that exert their antitumour activity by binding to tubulin, and inhibiting microtubule assembly and cell division. *Trastuzumab*-DM1 is a novel maytansanoid antibody-drug conjugate that has shown promising efficacy as first-line therapy in patients with HER2 (human epidermal growth factor receptor 2) - positive metastatic breast cancer in a recent Phase II clinical trial [35-49]. *Brentuximab* vedotin (SGN-35) is an immunoconjugate consisting of an anti-CD30 antibody and monomethyl auristatin E, an antimitotic agent which inhibits cell division by blocking the polymerization of tubulin. A recently published Phase I trial showed that *brentuximab* vedotin provided a high response rate in patients with relapsed or refractory CD30-positive lymphomas [50]. Figure 5 shows the sequence of events that occur upon binding of the ADC to the target antigen.

U. Iyer, V.J. Kadambi / Journal of Pharmacological and Toxicological Methods xxx (2011) xxx–xxx

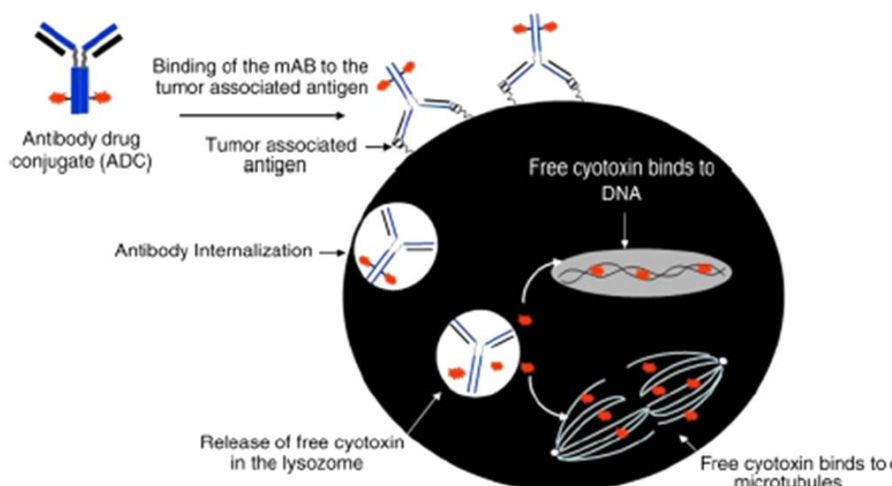


Fig. 5 Mechanism of Action of ADCs. The ADC is internalized by receptor-mediated endocytosis and cleavage of the linker occurs within the cell to release the cytotoxin, which then targets either the DNA or the microtubules to kill the cell (Iyer U., *et al.*, 2011).

Careful selection of the different components of the ADC is critical to successful design of ADCs that could be used as cancer therapeutics. Apart from optimizing the component parts, efforts to build better ADCs have led to much research into all the characteristics of the ADC that may affect its therapeutic index, including conjugation of the antibody to the linker and the drug, drug-antibody ratios, and the mechanism by which ADCs effect cell killing.

DIAGNOSTIC APPLICATION OF ANTIBODIES

Conventional tumor imaging approaches such as CT (contrast-enhanced helical) and MRI (magnetic resonance imaging) mainly focus on delineating morphological features of the tumor such as anatomic location, extent, and size. Despite continuous

improvements in spatial resolution with advanced imaging equipment, imaging modalities using non-targeted contrast agents, such as CT and MRI, have limited sensitivity and ability to provide specific and functional informations on the disease, which is increasingly recognized to be an obstacle to earlier diagnosis and the monitoring of treatment responses. Recent advances have stimulated the emergence of the new field of “molecular imaging,” which focuses on visualizing or imaging biological events and processes in living systems, including patients. Current molecular imaging approaches include scintigraphy/immuno-scintigraphy, PET/immuno-PET and optical imaging including fluorescence-mediated tomography and near-infrared fluorescence reflectance (NIRF) imaging.

The development of antibodies for molecular imaging has been promoted by the development of engineered fragments with pharmacokinetics optimized for imaging. Reduction of the overall size of antibody-based fragments can result in accelerated blood clearance due to removal of the Fc region and by reduction of the molecular weight to a size below the threshold for first-pass renal clearance. scFvs represent convenient modules for engineering antibody-based fragments that retain full binding specificity (i.e. diabodies, triabodies, tetrabodies, tandem diabodies and minibodies derived from the fusion of scFvs to the antibody CH3 domain) but also exhibit a suitable range of format dependent blood-clearance properties. Successful imaging requires that the tracer must circulate long enough to allow sufficient perfusion and binding in target tissues, coupled with reasonably fast clearance from the circulation. Diabodies, for example, exhibit terminal half-lives in the range of 3–5 h and can reach levels of 7%-

10% injected dose per gram in tumor xenograft models. Bivalent minibodies above the threshold for renal clearance exhibit intermediate kinetics and allow higher overall uptake levels (20% injected dose/g) in target tissues.

Radioimmunoscintigraphy

Radioimmunoscintigraphy (RIS) involves the administration of radiolabeled monoclonal antibodies (mAbs) directed against specific molecular targets, followed by imaging with an external gamma camera. MABs that react with specific cellular antigens are conjugated with a radiolabeled isotope. The labeled antibody-isotope conjugate is then injected into the patient and allowed to localize to the target over a 2-7 days period. The patient then undergoes imaging with a nuclear medicine gamma camera and radioisotope counts are analyzed. Imaging can be performed with planar techniques or by using single photon emission computed tomography (SPECT).

To date FDA-approved monoclonal antibody radiopharmaceuticals include (2009 CPT/HCPCS revisions):

- ¹¹¹I-*satumomab pendetide* (CYT-103, OncoScint CR/OV) has been FDA approved for imaging of colorectal and ovarian carcinoma;
- ⁹⁹Tc-*nofetumomab merpentan* (Verluma) is FDA approved for the detection of extensive stage disease in patients with biopsy confirmed, previously untreated small cell lung cancer patients;

Introduction – Cancer Immunotherapy

- ^{99}Tc - *arcitumomab* (IMMU-4, CEA-Scan) has been FDA approved for use in colorectal and ovarian carcinoma;
- ^{111}I -*pentetreotide* (Octreoscan) has been FDA approved for use in scintigraphic localization of primary and metastatic neuroendocrine tumors bearing somatostatin receptors, typically pancreatic islet cell tumors or adrenals ones;
- ^{111}I -*capromab pendetide* (Prostascint) has been FDA approved for imaging of pelvic lymph nodes in patients with newly diagnosed, biopsy proven prostate cancers or in post-prostatectomy patients in whom there is an high clinical suspicion of occult metastatic disease. ProstaScint consists of an intact murine monoclonal antibody, 7E11-C5.3, to which a linker chelator (GYK-DTPA-HCL) is bound, labeled with ^{111}In . The overall sensitivity and specificity of detecting disease using capromab pendetide has varied in reported studies. These studies demonstrated average sensitivities of 60%, specificities of 70%, positive predictive values of 60%, and negative predictive values of 70%. In a meta analysis, ^{111}In -labeled capromab pendetide studies in 2,154 patients from 15 institutions were analyzed. The overall sensitivity for detection of tumor in biopsy-proven primary carcinoma was 80%. The sensitivities ranged from 75% to 99% because of significant inter-reader variability. Antibody imaging is limited by its ability to detect viable disease. This may be related to the fact that the antibody binds to the intracellular part of PSMA and hence detects only necrotic tissue. J591, an antibody that detects the extracellular domain of PSMA, has been studied. These studies have

demonstrated antibody localization to known disease. However, this antibody has not been extensively studied as an imaging agent. [51-52-53-54].

Immuno-PET

Successful implementation of immuno-PET requires an appropriate match between the physical half-life of the positron-emitting radionuclides and biologic half-life of the antibody. PET radionuclides with half-lives in the order of days would be ideally suited for conjugation with intact antibodies for clinical imaging; recent examples are represented by ^{89}Zr (78.4 h) and ^{124}I (100.2 h). The clinical utility of ^{89}Zr -labeled U36, a chimeric antibody specific for CD44v6, has been demonstrated in a series of 20 patients with head and neck cancer. All of the primary tumors and 72% of the lymph node pathologist-confirmed metastases were detected in this clinical immuno-PET study. Similarly immuno-PET imaging, with ^{124}I -labeled cG250, an antibody directed against anticarbonic anhydrase-IX, resulted in successful detection of 15 of 16 clear-cell carcinomas. In these pre surgical studies, the final immuno-PET scans were acquired 6–8 days after injection, just before surgery. In order to further improve quality and specificity of PET-imaging other strategies are under study; pre targeting approaches in particular represent the new frontier also for imaging applications. Antibody loading of antigenic sites followed by radionuclide hapten delivery could allow higher tumor/blood localization ratio, lowering side effects due to longer half-life of pre-constituted antibody radionuclides complexes. One of the most promising developments, with implications for clinical translation, is the demonstration of rapid ^{18}F labeling and imaging of colon carcinoma xenografts pretargeted with an anti-CEA diabody. The

antigen-positive tumor was readily detected by small-animal PET imaging at time points from 1 to 6 h after injection [55].

Besides antibody and radionuclide features, development of immuno-PET strategies has also to face antigen characteristics such as internalization and normal vs tumor tissue expression. Selection of the appropriate receptor or antigen on cancer cells is crucial for the optimal design of targeted imaging approach. The ideal targets are those that are abundantly and uniquely expressed on tumor cells, but have negligible or low expression on normal cells. The targeted antigen should also have a high density on the surface of the target tumor cells. Whether the conjugates are prone to be internalized after binding to the target cell is another important criterion which addresses the choice of proper targeting strategy. In the context of pre-targeting RIT strategies, targeting internalizable antigens implies that the antibody-antigen complex is no longer available for subsequent tracer binding; this fact render internalizing antigens unsuitable for multisstep pretargeting approaches unless residualizing radiolabel measures are adopted. Conjugates directed against internalizing antigens, once internalized, are also addressed to intracellular processing which often lead to separation of vehicle from tracer [56].

Furthermore many targets that are suitable for therapeutic applications, such as epidermal growth factor receptor, are not likewise imaginable because of normal tissue expression.

Introduction – Mesothelin Targeted Cancer Immunotherapy

Mesothelin is a differentiation antigen whose expression in normal human tissues is limited to mesothelial cells lining the pleura, pericardium and peritoneum [57-58]. However, mesothelin is highly expressed in several human cancers, including virtually all mesotheliomas and pancreatic adenocarcinomas, and approximately 70% of ovarian cancers and 50% of lung adenocarcinomas [59-62] (Table 2). The mesothelin gene encodes a precursor protein of 71 kDa that is processed to a 31 kDa shed protein called megakaryocyte potentiating factor (MPF) and a 40 kDa fragment, mesothelin, that is attached to the cell membrane by a glycosyl- phosphatidylinositol (GPI) anchor [57-63] (Figure 6).

Table 2: Mesothelin expression in human cancers ^a

TUMOR	MESOTHELIN EXPRESSION (%)	COMMENTS	REFERENCE
Mesothelioma	100	Present in all epithelial mesotheliomas but absent in sarcoma type	59,64
Pancreatic cancer	100	Absent in normal pancreas and chronic pancreatis	60,62,70
Ovarian cancer	67-100	Mostly in serous ovarian adenocarcinomas although it is also expressed to a lesser degree in other sub- types	58,61,62,68
Lung adenocarcinoma	41-53	Some expression in squamous and large cell lung cancer but absent in small cell lung cancer	62,69

^aDetected by immunohistochemistry (Hassan R., *et al.*, 2008).

MESOTHELIN EXPRESSION IN HUMAN CANCERS

Mesothelin gene expression in human cancers has been studied using serial analysis of gene expression (SAGE) tag analysis (<http://www.ncbi.nlm.nih.gov/projects/SAGE/>). High mRNA expression of mesothelin is found in mesothelioma, lung, ovarian and pancreatic adenocarcinomas. In addition, immunohistochemistry (IHC) has helped delineate the frequency and pattern of mesothelin protein expression in these tumors (Figure 7). These studies have been greatly facilitated by the commercial availability of

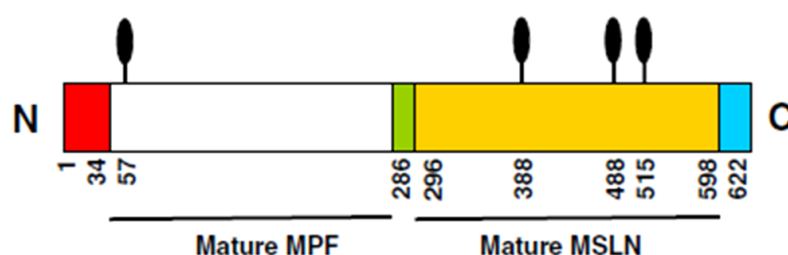


Fig. 6 Schematic of mesothelin. The human mesothelin (MSLN) gene encodes a precursor protein of 622 amino acids. On translocation into the endoplasmic reticulum (ER) the N - terminal signal peptide (red; residues 1–33) and the C - terminal glycosyl-phosphatidylinositol (GPI) anchor addition signal (blue; a predicted cleavage site: Ser598) are removed and the latter replaced with a GPI anchor. The MSLN precursor is cleaved into two products, mature megakaryocyte potentiating factor (MPF; residues Ser34– Arg286) and the GPI-anchored membrane-bound mature MSLN (orange) starting from Glu296. The proteolytic cleavage region (green) contains a furin cleavage site at Arg295, and other protease cleavage sites including a trypsin cleavage site at Arg286. The four N-linked glycans (black lollipop; Asn57, Asn388, Asn488 and Asn515) are indicated (Hassan R., *et al.*, 2008).

monoclonal antibody (mAb) 5B2 (Novocastra, Newcastle-on Type, UK) that can detect mesothelin expression in paraffin embedded tissues.

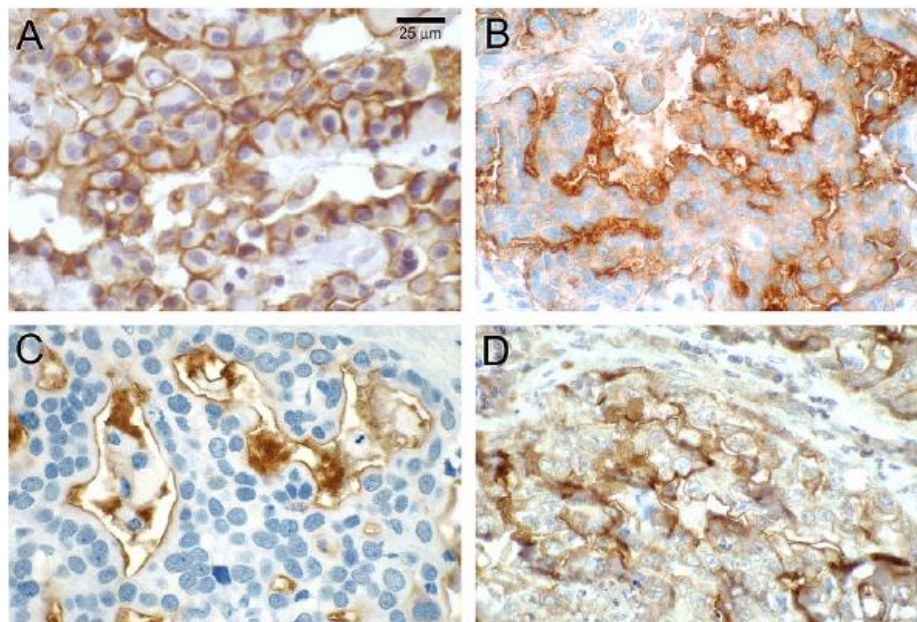


Fig 7 Mesothelin expression in human tumours. Mesothelin expression was detected by IHC using monoclonal antibody (mAb) 5B2 in tissue specimens of patients with mesothelioma (A); ovarian cancer (B); pancreatic adenocarcinoma (C) and lung adenocarcinoma (D) (Hassan R., *et al.*, 2008).

Mesothelioma

Mesothelin is highly expressed in epithelial malignant mesothelioma. In the original study by Chang and colleagues, mesothelin expression was evaluated by mAb-K1 using frozen section tissues of patients with malignant mesothelioma [64]. Out of the 23 pleural mesothelioma samples analysed all 15 epithelial mesothelioma samples had mesothelin expression, while 4 sarcomatous mesotheliomas were negative. In the four samples with biphasic mesothelioma, only the epithelial component stained for mesothelin. Mesothelin expression in paraffin embedded mesothelioma tissue samples was studied by Ordonez using mAb 5B2 [59]. Out of the 55 mesothelioma specimens

(44 epithelioid, 3 biphasic and 8 sarcomatoid) studied mesothelin reactivity was noted in all epithelioid mesotheliomas and the epithelial component of biphasic mesotheliomas. However, none of the sarcomatous mesotheliomas expressed mesothelin. These results are in agreement with the results of Chang and colleagues that mesothelin is present in all epithelial mesotheliomas and is absent in the sarcomatous type. Although mesothelin is not a specific marker for mesothelioma a negative mesothelin immunostain strongly argues against the diagnosis of epithelioid mesothelioma [65].

Pancreatic cancer

Argani and colleagues were the first to show mesothelin expression in pancreatic ductal adenocarcinoma [60]. Using SAGE database they found the tag for mesothelin to be consistently present in pancreatic cancer libraries but not in normal pancreas. In addition, mesothelin mRNA expression was present in 4 of 4 resected primary pancreatic cancers and by immunohistochemistry all 60 resected primary adenocarcinomas were mesothelin positive. These results were confirmed by Hassan and colleagues who showed that mesothelin was expressed in all 18 cases of pancreatic adenocarcinomas examined but absent in normal pancreas and in chronic pancreatitis [66]. Ordanez also showed mesothelin was expressed in majority of pancreatic adenocarcinomas, but was absent in islet cell tumors of the pancreas [62]. In addition to pancreas mesothelin is also highly expressed in other adenocarcinomas of the biliary tree such as gallbladder cancer, and tumors of the common bile duct [66-67].

Ovarian cancer

Using mAb-K1 Chang and colleagues demonstrated mesothelin expression in 10 out of 15 non-mucinous epithelial ovarian carcinomas while as it was absent in all 4 mucinous ovarian cancers examined [58]. Using 5B2 anti-mesothelin antibody Ordanez noted mesothelin expression in 14 of 14 serous, 3 of 3 endometrioid, 6 of 8 clear cell and 3 of 6 mucinous ovarian carcinomas [62]. Using tissue microarrays containing ovarian cancer specimens Frierson and colleagues showed that all 38 serous ovarian cancers expressed mesothelin while only 1 of 8 mucinous ovarian tumor had mild mesothelin expression [68]. In contrast to the studies of Ordanez and Frierson and colleagues who noted mesothelin expression in 100% of serous ovarian cancer, Hassan and colleagues noted mesothelin expression in 27 of 33 (82%) of serous ovarian cancers [61]. Based on these results one can conclude that mesothelin expression is present in most serous ovarian cancers, which constitute the majority of epithelial ovarian cancers, but is also expressed to a lesser degree in other subtypes of ovarian cancer.

Lung cancer

Though earlier studies using mAb-K1 showed no expression of mesothelin in lung adenocarcinomas, several recent studies using the 5B2 anti-mesothelin mAb show high expression in these tumors [64]. In the study by Ordanez looking at mesothelin expression in different human cancers, mesothelin expression was present in 14 of 34 (41%) lung adenocarcinomas [62]. This was confirmed in a large study by Miettinen and Sarlomo-Rikala who evaluated mesothelin expression in 596 lung carcinomas [73]. In this study, mesothelin expression was present in 78 of 148 (53%) adenocarcinomas, 15 of 118 (13%) large cell carcinomas, 29 of 124 (23%) squamous cell carcinomas and

0 of 41 (0%) small cell lung cancers. One unusual feature of the lung cancer is that there is more intracellular mesothelin reactivity than seen in mesotheliomas, ovarian or pancreatic cancer where the mesothelin staining is predominantly at the cell surface or normal mesothelial cells. Studies by Ho and colleagues have further delineated mesothelin expression in human lung cancer [70]. Using NCI-60 cell line panel they detected mesothelin mRNA in 7 of 9 (78%) lung cancer cell lines. In 4 of the 7 cell lines in which mesothelin mRNA was detected, cell surface mesothelin expression was detected by flow cytometry. In addition, mesothelin mRNA was present in 10 of 12 (83%) lung adenocarcinomas samples obtained from patients. These results support earlier studies by Ordanez and Miettinen that show mesothelin expression by immunohistochemistry in about half the patients with lung adenocarcinoma.

Mesothelin expression in other human tumors

In addition to the above tumors mesothelin over-expression has been noted in some other human cancers. It is commonly expressed in squamous cell carcinomas of different sites such as cervix, lung and head and neck carcinomas as well as endometrial adenocarcinomas [62-71-72]. Mesothelin expression by immunohistochemistry is infrequently present in colorectal, gastric and esophageal cancers [62]. Mesothelin expression is absent in soft tissue sarcomas with the notable exception of biphasic synovial sarcomas [62]. Cancer with absent mesothelin expression include melanomas, renal cell cancer, transitional cell carcinomas, thyroid cancer, breast cancer, prostate cancer and germ cell tumors [62].

MESOTHELIN BIOLOGY

The normal biologic function of mesothelin is not clear. Bera and colleagues generated mutant mice in which the mesothelin gene was inactivated, and neither mesothelin mRNA or protein was detected in the homozygous mutant mice [73]. These mesothelin knockout mice did not have a detectable phenotype and both males and females produced offspring normally. These results suggest that in mice mesothelin function is not essential for growth or reproduction. Although the functions of mesothelin remain largely unknown, recent studies have shed light on the possible role of mesothelin in cancer biology.

Mesothelin binding to MUC16/CA-125 and ovarian cancer

MUC16/CA125 is a very large cell surface mucin, with an average molecular weight between 2.5 and 5 million dalton, that is also heavily glycosylated with both O-linked and N-linked oligosaccharides [74]. It is shed into the serum and is used for monitoring response to therapy in ovarian cancer [75]. Rump and colleagues were the first to show that binding of CA-125 to membrane bound mesothelin mediates heterotypic cell adhesion since an anti-mesothelin antibody blocks this interaction [65]. Their results suggest that mesothelin is a novel CA-125 binding protein and that CA-125 might lead to intraperitoneal dissemination of ovarian carcinoma by binding to mesothelin present on normal mesothelial cells lining the peritoneal cavity. The biochemical basis of this interaction between mesothelin and CA-125 was further characterized by Gubbels and colleagues [76]. Using the MUC16 expressing ovarian cancer cell line OVCAR-3, they showed that it binds mesothelin while OVCAR-3 derived sublines that do not express MUC16 do not bind mesothelin. They also showed that mesothelin has a very strong

affinity for MUC 16 with an apparent K_d of approximately 5 nM and that mesothelin interacts with both soluble and cell surface associated forms of native MUC16. Taking together these studies provide evidence that mesothelin and MUC16 binding may be important in the peritoneal spread of ovarian cancer. Inhibiting MUC16 binding to mesothelin could therefore be a potentially useful strategy to treat ovarian cancer.

Mesothelin regulation by Wnt-1 and Wnt-5a

The Wnt ligands are secreted glycoproteins that play an important role in intracellular signalling and regulate a variety of biological processes including cell growth, cell differentiation and apoptosis. A mis-regulation of Wnt signalling has been shown to lead to the development of several human cancers [77]. Using the mouse mammary epithelial cell line C57mg, Prieve and Moon showed that mesothelin was up-regulated by Wnt-1 both by the stable expression of Wnt-1 in C57mg cells and as well as by co-culturing C57mg cells with Wnt-1 secreting cells [78]. They also demonstrated that mesothelin expression was induced by Li^+ , an inhibitor of GSK-3 β that mimics Wnt-1. In contrast to Wnt-1, Wnt-5a down-regulated mesothelin expression, perhaps through antagonism of endogenous Wnt/ β -catenin signalling. These results suggest that mesothelin expression can be altered by Wnt proteins. Interestingly, mesothelin is highly expressed in mesothelioma, lung, ovarian, and pancreatic carcinomas, which have constitutive activation of Wnt signaling [62-79].

Humoral anti-mesothelin immune response in cancer

To determine whether a spontaneous humoral B cell response to mesothelin is present in patients with mesothelin expressing cancers Ho et al used a sensitive enzyme-linked immunosorbent assay (ELISA) to detect mesothelin-specific IgG antibodies in serum of patients with advanced mesothelioma and ovarian cancer [80]. Elevated levels of mesothelin-specific antibodies were detected in the sera of 27 of 69 (39%) patients with mesothelioma and 10 of 24 (42%) patients with epithelial ovarian cancer when compared with a normal control population. Mesothelin specific antibodies were present at a higher frequency in patients whose tumors had strong mesothelin expression by immunohistochemistry. These results suggest that the immunogenicity of mesothelin is associated with its high expression on the tumor cells and serologic recognition of mesothelin is cancer related. The presence of a mesothelin specific B-cell response in a significant proportion of patients with mesothelin expressing tumors supports ongoing efforts to use mesothelin as a therapeutic cancer vaccine.

Prognostic significance of mesothelin expression in ovarian cancer

The significance of tumor mesothelin expression with clinical outcome in ovarian cancer was studied by Yen and colleagues [81]. In this study, tumor mesothelin expression by immunohistochemistry was correlated with clinical outcome in 198 patients with ovarian serous carcinoma. Mesothelin immunoreactivity was present in 55% of serous carcinomas with similar expression in both high grade and low grade tumors. The results of this study showed that in patients with high-grade advanced-stage ovarian cancer treated with optimal debulking surgery and chemotherapy, diffuse tumor mesothelin immunostaining correlated significantly with prolonged survival.

Mesothelin expression did not correlate significantly with patient age, tumor site, tumor grade, in vitro drug resistance and tumor cell differentiation. The authors speculate that a humoral or T cell immune response to mesothelin-expressing ovarian carcinoma cells could result in reduction of tumor load leading to the prolonged patient overall survival. However, the prognostic significance of mesothelin expression in ovarian cancer and other mesothelin expressing tumours needs to be validated in large prospective studies.

MESOTHELIN TARGETED THERAPIES

The limited expression of mesothelin on normal human tissues and high expression in several human cancers makes mesothelin an attractive candidate for cancer therapy. These therapies include agents that target cell surface mesothelin or elicit an immune response against mesothelin. Agents that are in the clinic or about to enter clinical trials include CAT-5001, MORAb-009 and CRS-207.

SS1P (CAT-5001)

SS1P is a recombinant immunotoxin consisting of an antimesothelin Fv linked to a truncated *Pseudomonas* exotoxin that mediates cell killing [82-83]. After binding to mesothelin, the immunotoxin is internalized via clathrin coated pits, undergoes processing in the endocytic compartment and the immunotoxin fragment containing the ADP-ribosylation domain is transported to the endoplasmic reticulum and then translocated to the cytosol where it inhibits elongation factor-2 leading to inhibition of protein synthesis and ultimately cell death [84]. Pre-clinical studies have shown that SS1P is cytotoxic to cell lines expressing mesothelin and causes complete regression of mesothelin expressing tumor xenografts in nude mice [63]. In addition SS1P is

cytotoxic to tumor cells obtained directly from human patients. Tumor cells obtained from patients with ovarian cancer undergoing surgery were grown in three dimensional organotypic cultures and treated with SS1P [85]. After treatment the organotypic gels were formalin fixed and evaluated for light microscopic examination and apoptosis. SS1P caused a dose dependent increase in tumor cell death and apoptosis. Similarly tumor cells established from ascites of patients with peritoneal mesothelioma are very sensitive to SS1P with an IC₅₀ of 0.08–3.9 ng/ml [86]. These studies show that mesothelin is highly expressed on tumor cells obtained directly from patients with mesothelioma and are very sensitive to treatment with SS1P. Recent studies have looked at the anti-tumor activity of SS1P in combination with radiation therapy or chemotherapy. Athymic nude mice bearing A431/K5 mesothelin expressing tumors were treated with radiation alone, SS1P alone or the two agents in combination [87]. The results of this study showed that mice treated with low-dose radiation and SS1P or high-dose radiation and SS1P had a statistically significant prolongation in time to tumour doubling or tripling compared with control, SS1P or radiation alone treated mice. Two Phase I studies of SS1P have just been completed. These studies which were designed to test the safety, maximum tolerated dose (MTD) and pharmacokinetics of SS1P used two different strategies for SS1P administration. In one study SS1P was administered as an intravenous bolus infusion over 30 min (SS1P bolus infusion study) while as in the other study SS1P was given as a continuous i.v. infusion over 10 days (SS1P continuous infusion study). Since SS1P given as a bolus infusion has a prolonged half life and can be given at much higher dose and several patients showed anti-tumor response, this is the schedule that is being pursued for its further clinical development. Based on pre-clinical studies in animal models that show marked synergy when SS1P is

combined with chemotherapy, clinical trials of SS1P in combination with chemotherapy are about to start for the treatment of mesothelin expressing malignancies.

MORAb-009

MORAb-009 is a high-affinity chimeric (mouse/human) monoclonal IgG1/ κ with high affinity and specificity for mesothelin. The heavy and light chain variable regions of mouse antimesothelin scFv (obtained by panning on mesothelin-positive cells a phage display library made from splenic mRNA of a mouse immunized with mesothelin cDNA) were grafted in frame with human IgG1 and κ constant regions. Since MORAb-009 is a chimeric antibody containing only the mouse sequences that recognize human mesothelin, it should be less immunogenic and allow repeated administration to patients. Laboratory studies show that MORAb-009 kills mesothelin expressing cell lines via antibody dependent cellular cytotoxicity (ADCC) and in addition it inhibits the binding of mesothelin to CA-125.

Based on these preclinical studies a Phase I clinical trial of MORAb-009 has been initiated (www.clinicaltrials.gov/ct/show/NCT00325494).

CRS-207

The rationale for mesothelin as a tumor vaccine is based on studies showing that mesothelin can elicit a strong CD8⁺ T cell response in patients [88]. One of the mesothelin cancer vaccines in advanced stages of clinical development is CRS-207 (LmDactA/DinIB/hMeso). This vaccine utilises a live attenuated strain of the bacterium *Listeria monocytogenes* (Lm), a facultative intracellular bacterium, as the vector [89]. The engineered vector CRS-100, has deletions of the two genes that encode the

virulence determinants actA and internalin B (inlB), which results in a greater than 1000-fold decrease in virulence compared to the wild type Lm. CRS-100 is currently undergoing Phase 1 testing in patients with carcinoma and liver metastasis [90]. CRS-207 is a live-attenuated Lm vaccine strain based on CRS-100 that encodes human mesothelin. Preclinical studies show that CRS-207 elicits human mesothelin-specific CD4⁺/CD8⁺ immunity in mice and in cynomolgus monkeys and exhibits therapeutic efficacy in tumor bearing mice [91]. A Phase I clinical trial of CRS-207 for the treatment of patients with mesothelin expressing cancers is about to commence.

Mesothelin cancer vaccines

The utility of mesothelin as a tumor vaccine came from a clinical trial conducted by Jaffe and colleagues that involved vaccination of pancreatic cancer patients with GM-CSF transduced pancreatic cancer cell lines [92]. Out of the 14 patients treated on this study 3 developed a post vaccination delayed-type hypersensitivity (DTH) response to the autologous tumor, that was associated with prolonged survival [92]. Subsequent immunologic studies showed that a strong and consistent induction of CD8⁺ T cell response to multiple HLA-A2, -A3, and -A24-restricted mesothelin epitopes occurred exclusively in the three patients who had developed a vaccine induced DTH response [88]. In another vaccine study, T-cell lines derived from the native or the agonist mesothelin epitope were shown to lyse mesothelin expressing and HLA-2 positive pancreatic cancer, ovarian cancer and mesothelioma cell lines [93]. These studies support the potential utility of mesothelin in peptide and/or vector-mediated immunotherapy protocols for the treatment of cancers that highly express mesothelin.

Mesothelin targeted ovarian cancer gene therapy

Pre-clinical studies have evaluated mesothelin as a target for adenoviral-mediated gene therapy. Adenoviruses containing the mesothelin promoter driving reporter gene expression were evaluated using established ovarian cancer cell lines and purified tumor cells obtained from patients [94]. These studies showed that the mesothelin promoter is transcriptionally active in ovarian cancer cells but has significantly reduced activity in normal control cells. Also in the liver, which does not express mesothelin, mesothelin promoter activity was low making it a useful promoter for gene therapy. The utility of mesothelin for transductional gene therapy, i.e. to direct gene therapy agents to targets highly expressed in specific tumors was also evaluated in this study. An adenovirus vector containing Fc-binding domain was conjugated to the mouse anti-human mesothelin mAb. This transductional targeting to mesothelin led to increased transduction rates in ovarian cancer cell lines as well as tumor cells obtained from patients. In contrast there was no increase in gene transfer rate using this construct in mesothelin negative human fibroblast cells or the teratocarcinoma cell line PA-1. These results show that mesothelin could be a potentially useful candidate for combined transductional and transcriptional adenovirus-based gene therapy.

Mesothelin as a target for radioimmunotherapy

Mesothelin as target for radio-immunotherapy was evaluated in vivo using nude mice bearing mesothelin expressing A431/K5 xenografts [95]. This study used a pre-targeting strategy that involves the administration of streptavidin (SA)-conjugated antibody to target the tumor, administration of a clearing agent to remove any circulating antibody-SA conjugate from the blood and then the injection of radiolabelled biotin that localizes

to tumor tissue bearing SA-conjugated antibody. Mice bearing A431/K5 tumors were first injected with the anti-mesothelin tetravalent single-chain Fv-streptavidin fusion protein (SS1scFvSA) followed 20 h later by a synthetic clearing agent to remove any unbound SS1scFvSA. This was followed 4 h later by the administration of radiolabelled ⁹⁰Y-1,4,7,10-tetraazacyclododecane- N,N',N'',N'''-tetraacetic acid (DOTA)-biotin. Pretargeted therapy of A431/K5 tumor with ⁹⁰Y doses of 11.2–32.4 MBq resulted in a dose-dependent tumor response. In mice that were treated with 32.4 MBq of ⁹⁰Y, 86% survived tumor free for 110 days compared to a median survival of only 16 days in the untreated mice. Such a pre-targeted approach could potentially be useful in the clinic and reduce non-specific bone marrow toxicity.

However, the high immunogenicity of SA, could limit repeated administration of this agent.

The generation of an immunotoxin involves the chemical coupling or genetic fusion of a cell-selective ligand with a complete toxin or a modified form of the toxin. The ligand provides the cell binding and internalizing property, whereas the toxin inhibits crucial cell function and causes cell death upon translocation to the cytosol. The ligand can be a recombinant antibody or an antibody fragment, growth factor, carbohydrate antigen or tumor-associated antigen. Bacterial toxins, such as *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT), and plant toxins, such as ricin, saporin, gelonin and poke weed antiviral protein, are used in immunotoxin constructs [96]. The advantages of this approach over chemotherapy are the selective delivery of drugs to tumors, thereby reducing systemic toxicity and increasing potency.

The first generation of immunotoxins, developed 35 years ago, employed chemical conjugates of antibodies with intact toxins, or toxins with attenuated cell-binding properties. Although they showed tumor regression in some lymphoma patients, they were typically ineffective because the constructs were heterogeneous, nonspecific and were too large to infiltrate solid tumors [97]. The next generation also employed chemical conjugation with deletions in native cell-binding domains, generating many more target-specific immunotoxins. Recombinant DNA techniques were applied in the production of third-generation immunotoxins to promote tumor specificity and penetration, and to reduce the cost and complexity of production. The cell-binding domain of the toxin is genetically removed and the modified toxin fused with a ligand or with DNA elements encoding the Fv portion of an antibody in these constructs. The light- and heavy-chain variable fragments are either genetically linked (scFv) [98-99] or held together by a disulfide bond (dsFv) [100]. Compared with the single-chain toxins (scFv), disulfide-stabilized Fv (dsFv) molecules did not aggregate, were stable and also

overcame the major obstacle of poor penetration into bulky tumor masses. These targeted toxins posed problems such as immunogenicity, nonspecific toxicity and instability [100]. The identification and mutation of B-cell epitopes in PE and their incorporation into immunotoxins reduced the immunogenicity of the toxin [101]. A new field of immunotoxins called bispecific immunotoxins (DT2219ARL) has also emerged recently – involving the simultaneous specific targeting of two targets on cancers for increased efficacy and reduced nonspecific toxicity [102]. Recent endeavors involve a new generation of immunotoxins in which the cytotoxic moiety is an endogenous protein of human origin, for example proapoptotic protein or RNase [103]. At present, only one agent, denileukin diftitox, which is a fusion protein of truncated DT and interleukin 2 (IL2), has been approved by the FDA [104]. Several similar fusion proteins are currently in clinical trials.

IMMUNOTOXIN DEVELOPMENT

A variety of toxins, mainly from plants, fungi or bacteria, have been characterized, structurally optimized for in vitro stability, activity and safety, and evaluated in vivo in animal studies and clinical trials. Among them, ricin, PE and DT are the most frequently used.

Immunotoxins with RNases as the cytotoxic moiety are recent examples aimed at reducing the immunogenicity. These toxins generally consist of several domains – the cell-binding or cell-recognition domain, the translocation domain, which enables release of the toxin into the cytosol, and the activity domain responsible for cytotoxicity.

In immunotoxin development the binding domain of these toxins is replaced by the cancer-cell-specific ligands.

The cancer-cell-specific ligands direct the internalization of the toxins via receptor-mediated endocytosis.

Upon internalization the catalytic domain of the toxin is cleaved in the late endosome and translocates to the cytosol leading to cell death by various mechanisms (Figure 8).

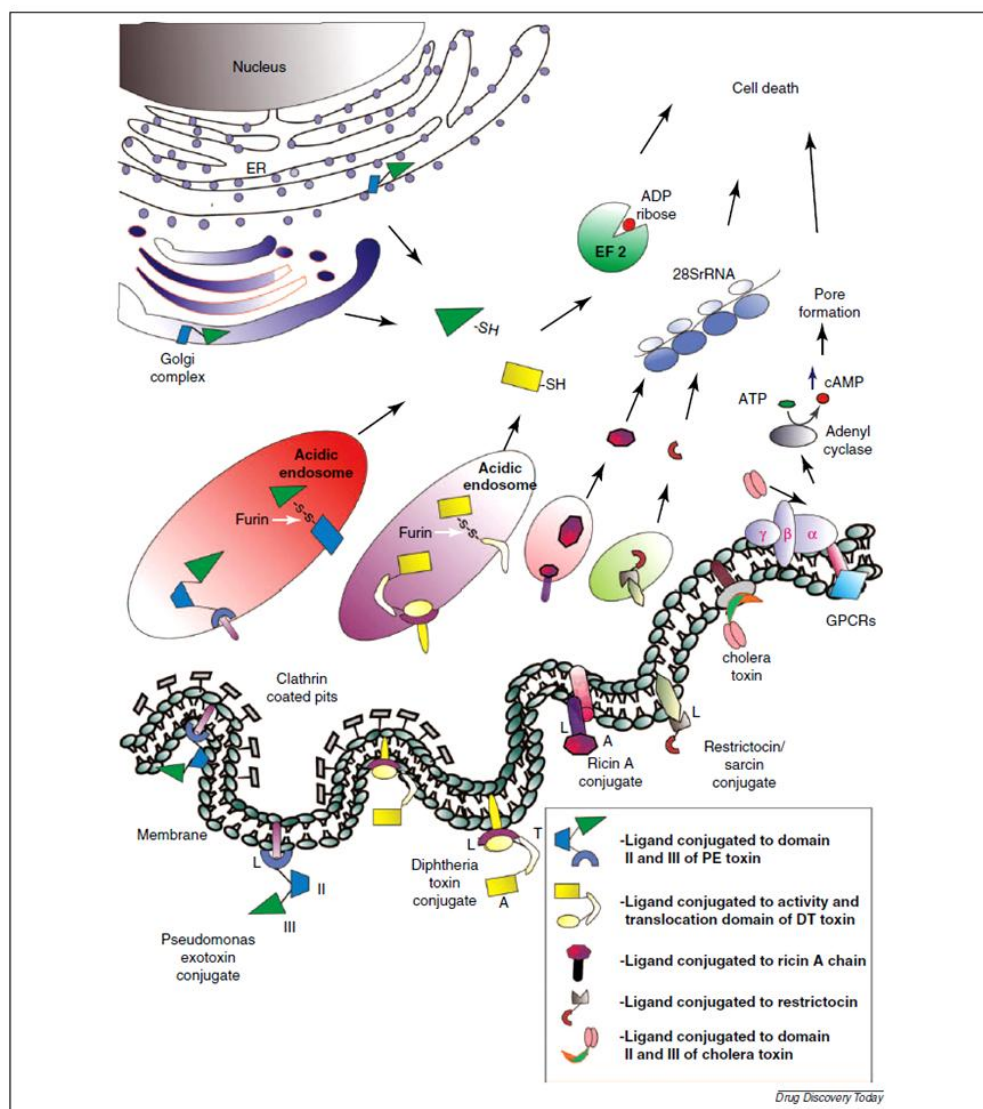


Fig. 8 Mechanism of action of immunotoxins. Immunotoxins enter the cell via receptor-mediated endocytosis. Upon binding to a cell-specific receptor, the toxin receptor complex is internalized through clathrin-coated pits into an endosome. For PE and DT, low pH induces unfolding of the protein, proteolytic cleavage and the release of the activity domain in the cytosol, where it inhibits protein synthesis by ADP ribosylation of the diphthamide residue of elongation factor 2 (EF2). Release of ricin in the cytosol leads to N-glycosylation of residues in 28S rRNA and prevents association of EF1 and EF2 with 60S ribosome, while restrictocin cleaves the 28S rRNA and leads to protein synthesis inhibition. Cholera toxin acts by ADP ribosylation of the Gs-α subunit of G proteins leading to an increased cAMP level and pore formation in the membrane resulting in cell death.

Table 3 represents the classification of clinically used toxins based on their mechanism of action. Many modifications in the toxin fragments have enabled improvements in cytotoxic activity and reduced immunogenicity over the years. The modified form of the toxins has also been identified in Table 3.

TABLE 3

Classification of toxins.

Toxins	Source	Mechanism	Structure	Modifications
ADP ribosylating toxins				
Diphtheria toxin	<i>Corynebacterium diphtheria</i>	ADP ribosylation of EF2	Activity (A chain), translocation (T) and binding (B) domains	a) DT ₄₈₆ b) DT ₃₈₈ or DT ₃₈₉ (deletion of cell-binding domain) c) CRM107 (point mutation in cell-binding domain of DT)
Pseudomonas exotoxin	<i>Pseudomonas aeruginosa</i>	ADP ribosylation of EF2	Binding (Ia) translocation (II and Ib) and activity domains (III)	a) PE40 and PE40KDEL b) PE38 and PE38KDEL c) PE38QQR d) PE35
Pore-forming toxins				
Cholera toxin	<i>Vibrio cholera</i>	ADP ribosylation of Gs- α subunit of G protein	Activity (A chain) and cell-binding (pentameric B chain)	CET40 (domains II and III)
Ribosome inactivating toxins				
Holotoxins – ricin	<i>Ricinus communis</i>	N-glycosylation of 28S rRNA	Activity and binding domain	a) Ricin b) Ricin chain A (RTA) c) bR (blocked ricin) d) dgA (deglycosylated ricin A-chain)
Hemotoxins – saporin (SAP), pokeweed antiviral protein (PAP)	<i>Saponaria officinalis</i> , <i>Phytolacca americana</i>	N-glycosylation of 28S rRNA	Single-chain proteins without binding domain	
Ribonucleases				
Fungal toxins – α -sarcin, restrictocin	<i>Aspergillus</i> sp.	Cleavage of 28S rRNA	Single-chain proteins without binding domain	
HPR, ECP, EDN	Human	Degradation of RNA	Single-chain proteins	

Abbreviations: DT: diphtheria toxin; DT388 or DT389: truncated forms of DT that lack receptor-binding activity; CRM107 or cross-reacting material – mutant of DT lacking receptor binding; PE: Pseudomonas exotoxin A; PE40 and PE38: truncated forms of PE that lack receptor-binding domain Ia; CET40: cholera exotoxin A; RTA: ricin toxin A; HPR: human pancreatic ribonuclease A; ECP: eosinophilic cationic protein; EDN: eosinophil-derived neurotoxin (Drug Discovery Today – Volume 16, Issues 11-12 – April 2011).

In this work of thesis our group decided to synthesize a chemical linked immunotoxin (IT) by use Ricin Toxin A (RTA). RTA belongs to Ribosome Inactivating Proteins (RIPs); the advantage of using RIPs is that rigorous purification to eliminate the B-chain

is not necessary. Moreover, no authors described an anti mesothelin antibody chemical linked to RTA to target such cancers.

RIBOSOME INACTIVATING PROTEINS BASED IMMUNOTOXINS

RIPs are a group of proteins that share the property of damaging ribosomes in a reversible manner, acting catalytically, i.e enzymatically. RIPs have been used to prepare immunotoxins or other conjugates, either by chemical linkage or as recombinant fusion proteins mostly with monoclonal antibodies but also with other suitable carriers, e.g. hormones (hormonotoxins), cytokines, neuropeptides. Currently, RIPs are divided into two groups, type 1 RIPs, consisting of a single peptidic chain of 30 kDa, approximately, and type 2 RIPs which, as found by Olsnes and Pihl (1973), consists of an enzymatically active A chain similar to type 1 RIPs, linked to a slightly larger (35 kDa, approx.) B chain, which has the properties of a lectin with specificity for sugars with the galactose structure [105]. It was proposed to include in a type 3 group two other proteins. One is a maize b-32 RIP, which is synthesized as a proenzyme, activated after the removal of a short internal peptide segment leaving two segments of 16.5 and 8.5 kDa [106]. The other one is JIP60, a RIP from barley in which a segment similar to type 1 RIPs continues with another segment of similar size without a known function [107]. More recently, it was suggested to include only the latter into this group [108]. A schematic representation of RIPs structure is in Figure 9.

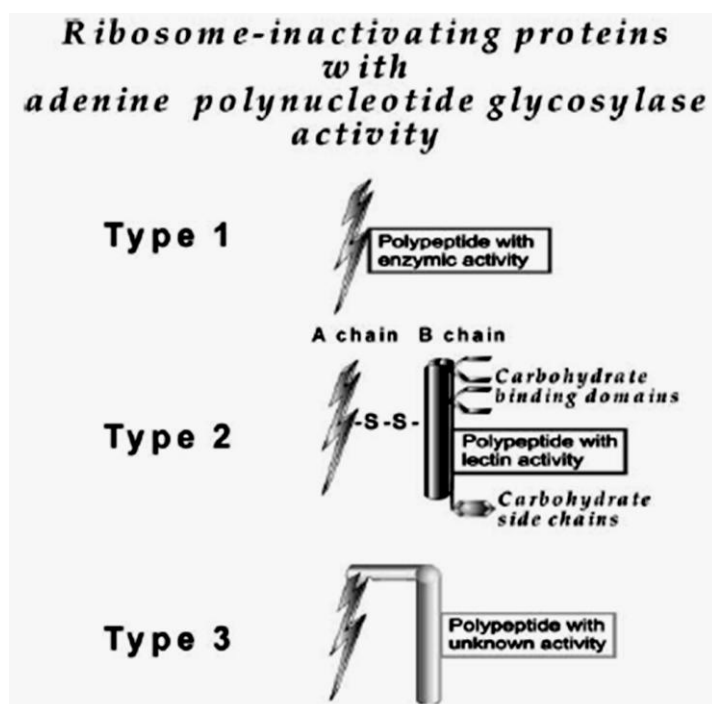


Fig. 9. Schematic representation of the structure of ribosome-inactivating proteins (Stirpe F., *et al.*, 2004).

Distribution in nature and mechanism of action

RIPs were initially detected in plants, mostly in Angiosperms, both mono- and dicotyledons, and also in mushrooms [109-110], and in an alga, *Laminaria japonica* [111]. The level of RIPs in plant tissues is highly variable, ranging from traces to hundreds of milligrams per 100 g. In some plants, RIPs are present in many or even in all tissues examined (roots, leaves, stems, bark, flowers, fruits, seeds, latex, cultured cells), whilst in other plants they are confined to a single tissue. RIPs are not limited to plants and an enzymatic activity similar to that of RIPs was detected in animal cells and tissues which, like in plants [112], was higher in virally-infected and stressed cells [113].

The mechanism of the ribosomal damage was discovered by Endo et al. (1987), who found that ricin cleaved the glycosidic bond of a single adenine residue (A₄₃₂₄ in rat liver rRNA). This residue is adjacent to the site of cleavage of rRNA by α -sarcin, in a

tetranucleotide GA₄₃₂₄GA in a highly conserved loop at the top of a stem, for this called α -sarcin/ricin loop (Figure 10). This observation was extended to other RIPs [114], which were officially classified as rRNA N-glycosidases (rRNA N-glycohydrolases, EC 3.2.2.22).

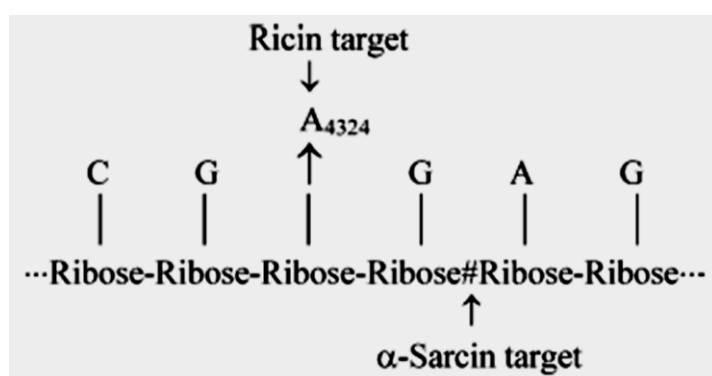


Fig. 10 Schematic representation of the enzymatic action of ribosome-inactivating proteins on rRNA, RIPs cleave a single adenine base (A₄₃₂₄ in 28 S rat mRNA) at a site adjacent to the site of attack by α -sarcin, which cleaves the phosphodiester bond between G₄₃₂₅ and A₄₃₂₆ in rat 28 S rRNA.

Subsequently, it was found that (i) some RIPs remove more than one adenine residue per ribosome [115] and (ii) that RIPs remove adenine residues from DNA and other polynucleotides [116-117-118].

A summary of the properties of RIPs is given in Table 4 (Stirpe F., *et al.*, 2004).

Properties of ribosome-inactivating proteins					
RIPs	Structure	Molecular mass (kDa)	Inhibition of protein synthesis (IC ₅₀ ^a , nM)		Toxicity to mice (LD ₅₀ , mg/kg)
			Cell-free ^b	HeLa cells	
TYPE 1	One chain	26–32	< 0.01–4.0	170–> 3300	0.95–44
TYPE 2	Two chains	60–65	43–88	0.0003–1.7	0.0017–0.008
Toxic Reduced			0.1–3.5		
Non-toxic Reduced	Two chains	56–63	0.6–> 100 0.05–7.4	0.54–> 15,000	1.4–> 40

^a Concentration causing 50% inhibition of protein synthesis.
^b In a rabbit reticulocyte lysate system.

Toxicity

Until recently it was assumed that all type 2 RIPs were potent toxins. The high toxicity was explained by the binding of the B chain to the surface of most cells. This occurs through binding of the lectin site to galactosyl-terminated residues on most cells. Once bound to cells, the B chain allows and facilitates the entry of the toxins in the cells, where the A chain can exert its enzymatic activity, damaging ribosomes and possibly other structures, with consequent cell damage and death.

Type 1 RIPs, being devoid of a B chain, enter with difficulty into cells and consequently are much less toxic than type 2 RIPs, but become highly toxic if they are introduced into cells by linkage to an appropriate carrier capable of binding to cells.

The cytotoxicity of RIPs is commonly attributed to the inhibition of protein synthesis consequent to ribosomal damage. However, this view was challenged when it was found that ricin induced the expression of Apo2.7 [119] and Bak [120], suggesting that de novo protein synthesis occurred in the apoptotic process triggered by the toxin. Furthermore, it was observed that ricin and Shiga toxin cause disruption of DNA in human endothelial (HUVEC) cells, in a manner that appeared before the activation of caspase 3 [121]. This alteration occurred simultaneously to protein synthesis inhibition in the case of ricin, and shortly afterwards in the case of Shiga toxin, and was not observed when a comparable inhibition of protein synthesis was induced with cycloheximide. Furthermore, saporin caused damage to genomic DNA [122], and it was concluded that both the rRNA glycosidase activity and the DNA internucleosomal fragmentation contributed to cell death. All this suggests that multiple effects of RIPs on nucleic acids may concur to the pathogenesis of cell damage.

Immunotoxins and other conjugates

RIPs of both types have been used, although toxic type 2 RIPs as such are not suitable, because their B chains would bind unselectively to virtually any cell. This difficulty has been circumvented either by the use of isolated A chains, that of ricin being the most commonly employed, or, less frequently, with the use of 'blocked ricin', in which galactose-binding sites are modified and rendered ineffective, or with the use of nontoxic type 2 RIPs, e.g. ebulin 1 [123]. Immunotoxins have been prepared also with type 1 RIPs, especially PAP, saporin and gelonin.

Most immunotoxins have been prepared for the experimental therapy of malignancies, for immunosuppression and also for the therapy of viral diseases. For these purposes RIPs have been linked to antibodies against antigens expressed prevalently on cancer cells [124] or on endothelial cells of tumor vasculature [125-126], on immunocompetent cells [127] or on cells infected by viruses, especially by HIV [128-129]. Immunotoxins work very well in vitro, in that with them, at appropriate concentrations, it is possible to eliminate target cells without too much damage to other cells, and under this respect they seem to be most valuable tools to remove contaminating cells from cell cultures.

In medicine, studies have been done to use immunotoxins in vitro to purge bone marrow suspensions from malignant or immunocompetent cells but, again, their use in therapy is not common.

A number of experimental studies have been done with tumors transplanted in animals. Results have been very encouraging, especially when treatment was started soon after the inoculum of tumors [130], suggesting that immunotoxins are more efficient on small cell masses. Consistently, better results were obtained with haematological than with

solid tumors, suggesting that a first difficulty with immunotoxins in vivo is their arrival to, and entry into, tumor cells.

Other applications were envisaged. Among these, immunotoxins were prepared with ricin [131] or saporin [132] and antibodies against the acetylcholine receptor.

These immunotoxins selectively damage muscles and are proposed, as an alternative to botulinum toxin, for the therapy of spasm or of strabismus [133-134-132].

A conjugate of saporin with substance P has been developed and proposed for the study and therapy of pain [135]. Clinical trials with immunotoxins were performed, most of them for treatment of cancer [136-124].

The results were encouraging, but still some problems prevent the use of immunotoxins in human therapy. As a whole, the side effects they cause are not worse than those of many conventional chemotherapy treatments, and include fever, fatigue, myalgia, and capillary leak syndrome. Repeated administration of immunotoxins is prevented by the immune response they elicit in the recipient organism.

Currently, this is a major obstacle to the therapeutic use of immunotoxins, which hopefully will be reduced, if not prevented, either by better immunosuppressive treatments, or by the use of humanized antibodies and possibly of recombinant human enzymes

AIM OF THE THESIS

Aim of this thesis was to develop new mAbs direct to the tumor associate antigen (TAA) mesothelin. Mesothelin is overexpressed in a variety of cancers including mesothelioma, ovarian cancer and pancreatic cancer. In addition, mesothelin is expressed on the surface of many lung adenocarcinomas and other types of lung cancer.

The generation of new mAbs mesothelin targeting can be a promising approach for therapeutic anti cancer applications (based on immunoconjugates spread toxin or chemotherapeutic agents) and diagnostic for imaging applications (by means of radioactive or fluorescence trackers).

Presently, drugs that are in the clinic or about to enter clinical trials for treatment of mesothelin overexpressing tumors include SSP1 (CAT-5001), that is a recombinant immunotoxin consisting of an antimesothelin murin scFv linked to a truncated *Pseudomonas* exotoxin A of *Pseudomonas Aeruginosa* and MORAb-009 that is a chimeric (mouse/human) mAb containing the same murin scFv of SSP1.

Our group decided to produce new mAbs recognizing mesothelin for these reasons:

- mAb to mesothelin is not already available, due to patent restrictions;
- potential availability of almost infinite quantities of a specific monoclonal antibody directed toward a single epitope;
- mAb cDNA can be used to engineer the protein to create single chain-variable fragments (scFvs), multivalent scFvs (diabodies, tribodies), minibodies (scFv-CH3 dimers), bispecific antibodies;
- mAb could be used for emerging nanoparticle technologies;

- production of chimeric and humanized mAbs to reduce the immunogenicity and to increase the immune system activation (ADCC antibody-dependent cell cytotoxicity and CDC complement-dependent cytotoxicity).

My work describes the development of a new anti-mesothelin mAb and the following functional characterization. The mAb that I have produced was obtained by hybridoma technologies. This antibody was tested by flow cytometry to evaluate the specificity and affinity for the antigen mesothelin. Subsequent goal of this project was to synthesize a chemical linked IMMUNOTOXIN (IT) conjugating the mAb anti mesothelin with the powerful plant toxin Ricin A (RTA) and to study the effects of this IT on mesothelin positive and negative cell lines by XTT assay.

MATERIAL AND METHODS

PLASMID pcDNA3.1 STRUCTURE

The pcDNA3.1(+) (Invitrogen) is a vector of about 5400 bp (Figure 11) for constitutive, high level expression of recombinant protein in mammalian cells. This plasmid contains a promoter sequence deriving from Cytomegalovirus (CMV) for a useful transcription of the cloned gene, and a bovine growth hormone (BGH) polyadenylation signal for the efficient transcription termination and polyadenylation of mRNA. It contains T7 promoter/priming site that allows *in vitro* transcription in the sense orientation and sequencing through the insert multiple cloning site in forward or reverse orientation that makes insertion of your gene possible and facilitates cloning. It presents pUC origin for high-copy number replication and growth in *E. coli*. The vector is composed also by the f1 (phago filamentous) origin that consents rescue of single-stranded DNA. Moreover, it is made up by SV40 early promoter and origin which allow efficient high-level expression of the neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen. It presents also SV40 early polyadenylation signal for a useful transcription termination and polyadenylation of mRNA. Finally, pcDNA3.1 is made of Ampicillin resistance gene (β -lactamase) for selection of vector in *E. coli* and neomycin resistance gene for the choice of stable transfectants in mammalian cells.

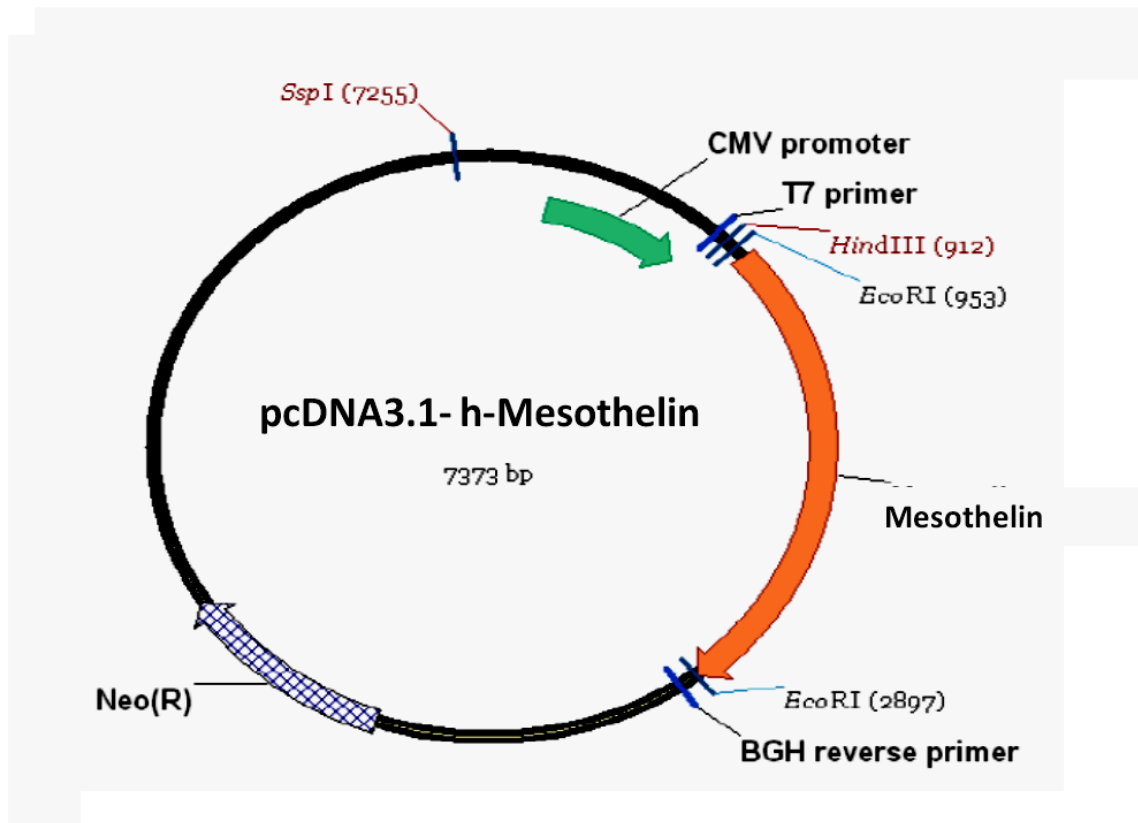


Fig. 11 The figure summarizes the features of the pcDNA3.1(+) vector (www.invitrogen.com).

CELL LINES

The cell lines were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). These cell lines included:

- Human embryonic kidney cell-line (HEK293);
- Human ovarian carcinoma cell line (OVCAR-3);
- Rat glial tumor cell line (C6);
- Human leukemic monocyte lymphoma cell line (U937);
- Human T cell lymphoblast-like (lymphoma) cell line (Jurkat);
- Human prostate cancer cell line (PC3);
- Human epithelial carcinoma cell line (A431);
- Human acute lymphoblastic T cell leukemia (CEM);

Material and Methods

HEK293 cell line transfected with mesothelin (HEK293-mesothelin) was produced in our laboratory by the transfection into HEK293 of pcDNA3.1-mesothelin. The cells were cultured in D-MEM (Dulbecco's Modified Eagle Medium, Biochrom AG, Berlin, Germany) or in RPMI-1640 supplemented with 10% FBS (Fetal Bovin Serum), L-Glutamine 2mM, penicillin–streptomycin 100 U/ml, HEPES 10 mM. The medium of the HEK293 was added with 500 µg/ml of G418 (Geneticin® reagent is an aminoglycoside related to Gentamicin and it is commonly used as a selective agent for eukaryotic cells, Gibco). Cells were then incubated at 37 °C with 5% CO₂ in a humidified atmosphere.

PRODUCTION OF RECOMBINANT MESOTHELIN

pET30a - mesothelin vector was transfected into the host strain, *E. Coli* BL21 DE3. The bacteria were triggered overnight in 100 ml of Lysogeny broth (LB) - plus kanamycin (1:1000), a nutritionally rich medium. 20 ml of this pre-culture were grown at 37°C in 1 liter of medium (in a stirrer) and they were induced in the logarithmic phase at OD_{600nm} value = 0,7 – 0,8 with 1 mM isopropyl β-D-thiogalactoside (IPTG) and cultured at 37°C for 3 h. The recombinant protein was obtained as cytoplasmic inclusion bodies. At this stage, the bacteria were centrifuged, and the pellet was lysated by sonication in inclusion bodies (IB) buffer (Tris 20 mM pH 7.5, EDTA 10 mM and Triton X-100 1%, lysozyme, Dnase I, PMSF and complete protease inhibitors). Cytoplasmic inclusion bodies' pellet was washed twice with IB buffer and then solubilized at 20 mg/ml in a solubilization buffer (CAPS 10X, DTT 1mM, N-lauroylsarcosine 1,2%).

Protein refolding method was obtained by sequential dialysis steps at 4°C:

Material and Methods

- 1st and 2nd steps dialysis (Volume = 50X) Tris HCl 1 M pH 8.5 + DTT 0.1 mM + PMSF 0.1 mM;
- 3rd and 4th steps dialysis (Volume = 50X) Tris HCl 1 M pH 8.5 + PMSF 0.1 mM;
- 5th step dialysis (Volume = 25X) Tris HCl 1 M pH 8.5 + PMSF 0.1 mM + L-Arginine 0.4 M + GSSG 0.5 mM + GSSH 5 mM;
- 6th dialysis (V = 100X) TP 5mM PH 8.0.

The purification was achieved by affinity chromatography taking advantage from the affinity of the imidazole rings of poly – histidine tails of His-tagged recombinant mesothelin for the resin Ni-NTA *His Bind Resin* (Novagen). The protocol was used as follows:

- Ni-NTA resin (column) was equilibrated with binding buffer (TP 5 mM pH 8.0);
- After centrifugation, the sample was incubated overnight with the resin and then filtrated twice through the column;
- Three washes were performed with wash buffer (TP 5 mM pH 8.0 imidazole 10 mM) to eliminate possible bacteria proteins (these proteins bind the resin in a not specific way);
- Finally, recombinant protein was eluted repeatedly with elution buffer (TP 5mM pH 8.0 imidazole 250 mM). The elutions were analyzed by spectrophotometer reading and polyacrylamide gel electrophoresis to establish the proteic content.

The validation of the production of a protein dimensionally corresponding to the mesothelin was obtained by polyacrylamide gel electrophoresis, whereas the definitive confirmation of the proteic identity was achieved through western blot (WB).

TRANSFECTION

The transfection was performed with LIPOFECTAMINE 2000 (Invitrogen), that is a reagent of transfection containing cationic liposome. The HEK293 wt cells were transfected with pcDNA3.1 containing the gene that expresses the mesothelin.

The cells were seeded in a 24-wells plate and the transfection was performed as follows (manufacturer protocol):

- the day before transfection 1.5×10^5 cells/well in 0.5 ml of complete growth medium were seeded and they were placed in incubator overnight (cell density should be 50-80% confluent on the day of transfection);
- the day of transfection, the DNA of interest (0.3 μ g) was diluted in 250 μ l of Opti-MEM medium (Invitrogen) for each well of cells to be transfected, as well as 1 μ l of Lipofectamine was added into the above diluted Opti-MEM-DNA solution, mixed gently and incubated for 30 minutes at room temperature to form DNA/Lipofectamine complexes;
- subsequently, the medium of growth was removed from the wells and the complexes DNA/Lipofectamine was adjoined directly to each well containing cells. Opti-MEM medium was added to each well until the total volume was of 500 μ l/well. The cells were incubated at 37°C in a CO₂ incubator for about 6 hours and then 2 ml/well of complete D-MEM were adjoined. The reaction lasted overnight.
- next morning, for each well of cells G418 antibiotic (500 μ g/ml) was added for the selection of cells steadily transfected;

After 48 hours from the transfection, FACS analysis was performed to evaluate the cells expressing the construct of interest.

BALB/c MICE IMMUNIZATION FOR ANTI-MESOTHELIN ANTIBODY PRODUCTION

Immunization protocol involves the preparation of antigens samples and their safe injection into laboratory or farm animals so as to evoke high-expression levels of antigen-specific antibodies in the serum. BALB/c immunization was performed by injection of cells membranes that overexpress the antigen mesothelin. The first immunization (day 0) was done with HEK293-mesothelin cells membranes by an intra-peritoneal (i.p) injection with about 500 µg of total membrane proteins suspended again in 400 µl of physiologic solution (NaCl 0.9%) emulsified in complete Freund's adjuvant (Sigma Aldrich). After 7 days (day 7), a booster was performed with OVCAR-3 cells membranes (i.p injection) emulsified in Freund's incomplete adjuvant. After 10 days (day 10), the serum was recovered from the animal to evaluate the presence of anti-mesothelin antibodies. It is important to remind ourselves here that antisera generated in this way also contained considerable amounts of other antibodies directed against a variety of membrane antigenic determinants (these antisera were said to be polyclonal). The presence of anti-mesothelin antibodies was confirmed by FACS analysis on mesothelin positive cells and mesothelin negative cells. Subsequently, the final boost was performed by a combination of two immunizations at the same time: an intravenous (i.v) injection of 10 µg of recombinant mesothelin and an i.p injection with cells membranes of HEK293-mesothelin at -3, -2, -1 days from the splenectomy. The final boost was used for two purposes: to induce a good strong response and to synchronize the maturation of the response. This boost was done at least 3 weeks after the previous injection. This interval allowed most of the circulating antibodies to be cleared from the blood stream by the mouse.

ADJUVANTS

To enhance the immune response to an immunogen, various additives called adjuvants can be used. When mixed and injected with an immunogen, an adjuvant will enhance the immune response. This is not a substitute for a carrier protein, because it enhances the immune response to immunogens but cannot itself make haptens immunogenic. Adjuvants are nonspecific stimulators of the immune response, helping to deposit or isolate the injected material and causing a dramatic increase in the antibody response. There are many popular adjuvants, including Freund's complete adjuvant (FCA). This reagent consists of a water-in-oil emulsion and killed *Mycobacterium*. The oil-and-water emulsion localizes the antigen for an extended period of time, and the *Mycobacterium* attracts macrophages and other appropriate cells to the injection site. Inject Freund's Complete Adjuvant is used for the initial injections. Subsequent boosts use immunogen in an emulsion with inject Freund's Incomplete Adjuvant, which lacks the *Mycobacterial* component. Freund's adjuvants are very effective, but they do pose risks to both animal and researcher because of the toxic *Mycobacterial* components.

PREPARING MACROPHAGE FEEDER CELL CULTURES

The macrophages were isolated from the peritoneal cavity of a mouse washing the cavity with RPMI-1640 medium cold (to promote macrophage detachment). The macrophages diluted in RPMI-1640 were seeded in a 24-wells plate and incubated at 37°C in a 5% CO₂ incubator.

After 12 hours, each well was washed with RPMI-1640 and the medium of incubation was substituted with fresh complete RPMI-1640 medium. The macrophages were irradiated with a Cs source irradiator at 6000 rad. In this way, the macrophages lose the

Material and Methods

ability to proliferate even if they are able to secrete cytokines that stimulate the growth of the hybridomas.

PREPARING SPLENOCYTES FOR FUSION

The mouse was sacrificed. The spleen was removed aseptically from an immunized animal and placed in a 100-mm tissue culture dish containing 10 ml of medium without serum (RPMI-1640). Contamination tissue was trimmed off and discarded from the spleen.

The spleen was teased apart using 19-gauge needles on 1.0-ml syringes (continue to tease until most cells have been released and the spleen has been torn into very thin parts). Cell clumps were disrupted by pipetting. The cells and medium were moved into a sterile centrifuge tube (on ice). In this way the cells were transferred leaving behind larger pieces of tissue. The tissue culture plate and tissue clumps were washed with 10-ml of medium without serum (RPMI-1640) and combined with the first 10 ml in the tube.

The cell suspension was allowed to sit for approximately 2 min. This permitted larger cell clumps to settle to the bottom of the tube. The supernatant was carefully removed from the sediment and transferred to a fresh centrifuge tube.

A spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

PREPARING MYELOMA CELLS FOR FUSION

Myeloma cells were thawed from liquid nitrogen stocks at least 6 days prior to the fusion. The myeloma cells were grown rapidly and healthy before the fusion in

complete RPMI-1640 20% FCS (fetal calf serum). A day before, the cells were splitted into fresh medium at a final concentration of 5×10^5 cells/ml. In this way, the morning of the fusion myeloma cells were at least 2×10^7 .

FUSION PROTOCOL

The technique of hybridization of an immortal myeloma cell line with an antibody-producing B cell, as developed by Kohler and Milstein in the late 1970s, provided a technique by which monoclonal antibodies could be produced in virtually unlimited quantities. Since the technique involved the selection of single cells and the clonal expansion of a single hybrid between the antibody-forming cell and the myeloma cell, it made available, for the first time, monoclonal antibodies for usage in cancer biology (Figure 12).

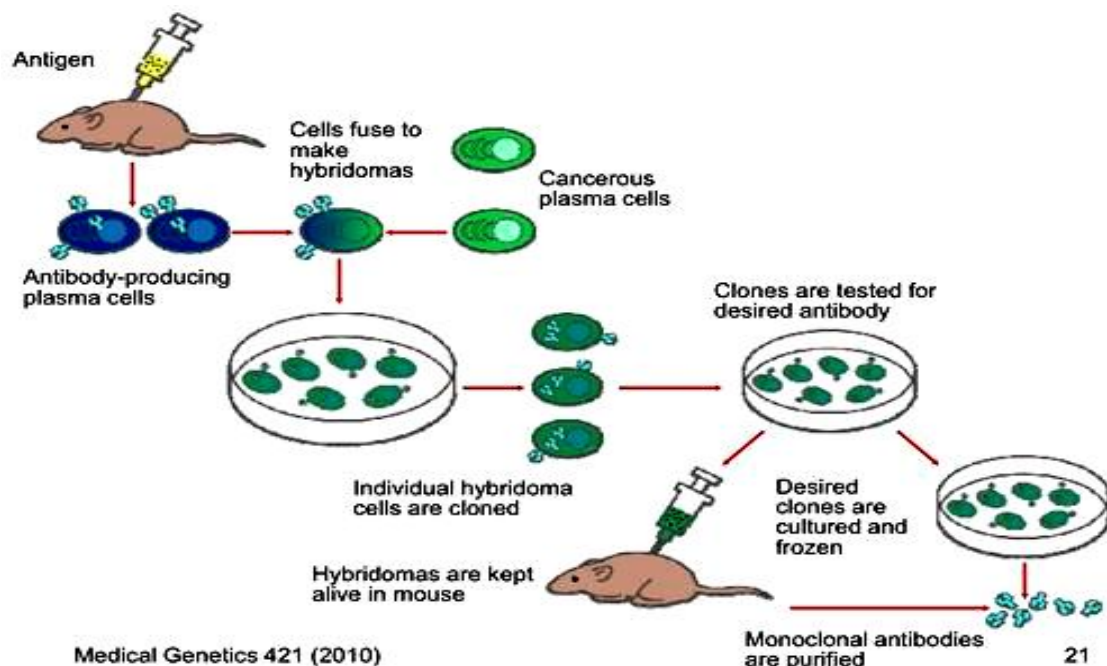


Fig. 12 Production of mAb: use of hybridoma technology.

Material and Methods

Cell fusion induced by polyethylene glycol (PEG) has become a standard method in somatic cell genetics. PEG promoting cell fusion is also the standard procedure for the production of hybridoma cells.

Myeloma cells and splenocytes were mixed in a ratio 1:7, respectively. The cellular suspension was centrifuged at 250 x g for 10 minutes at 20°C.

After the centrifuge, the supernatant (medium) was carefully removed as completely as possible with a Pasteur pipette. A complete removal of the supernatant is essential to avoid dilution of PEG. The pellet was broken by gently tapping the bottom of the tube. The tube was placed in a 37°C water bath and kept there during the fusion.

1 ml of 50% PEG 1500 pre-warmed was added to the pellet using 1 ml pipette, over a period of 1 minute, constantly stirring the cells with the pipette tip.

Stirring the cells in 50% PEG 1500 was carried on for a further 1-2 minutes.

1 ml of medium without serum (RPMI-1640) pre-warmed at 37°C was adjoined to the fusion mixture, continuously stirring as before, over a period of 1 minute.

3 ml of medium without serum (RPMI-1640) pre-warmed at 37°C was added over a period of 3 minutes, continuously stirring the cells.

10 ml of medium without serum (RPMI-1640) pre-warmed at 37°C was adjoined slowly and the pellet was incubated for 5 minutes at 37°C.

The cells were centrifuged and the supernatant was discarded. The pellet was suspended again in complete RPMI-1640 20% FCS.

The cellular suspension was seeded in the 24-wells plate previously treated with macrophage irradiated (see p. 53 “preparing macrophage feeder cell cultures”) and incubated at 37°C in a 5% CO₂ incubator.

Material and Methods

After 24 hours, the medium of incubation was added with hypoxanthine-aminopterin-thymidine (HAT - Sigma) supplemented medium. Aminopterin blocks the synthesis of DNA by inhibiting dihydrofolate reductase. Cells that lack the ability to use the rescue pathway for nucleotide synthesis are eliminated. Cells that possess hypoxanthine – guanine phosphoribosyl transferase (HPRTase) and thymidine kinase (TK) enzymes can exploit the salvage pathway if supplied with hypoxanthine and thymidine. The purpose of the medium was: (1) selectively kill unfused myeloma cells that are well adapted to the tissue culture and that would otherwise outgrow any hybridomas produced and (2) eliminate any myeloma-myeloma hybridomas that lack HPRTase. HPRTase positive spleen-spleen hybridomas, although not sensitive to aminopterin, are normally short-lived in culture. In this way, only the B cell-myeloma hybrids survive. These cells produce antibodies (a property of B cells) and are immortal (a property of myeloma cells). Wells containing hybridomas were ready to start screening 14 days after the fusion. The first step was to collect the supernatant from the wells having hybridomas density 80-100% confluent and to test this supernatant by FACS analysis on mesothelin positive cells and mesothelin negative cells, to evaluate the presence of anti- mesothelin antibodies. Hybridomas positive to the screening (at this stage they were said to be polyclonal hybridomas) were cloned by limiting dilution in 96-wells plate. Because hybridoma cells have a very low plating efficiency, single cell cloning was done in the presence of macrophage feeder cells. After selection is complete (about 14 days), the supernatant of the hybridomas was tested by FACS analysis to verify the presence of hybridomas producing anti-mesothelin antibodies (at this stage they were said to be monoclonal hybridomas). Positive hybridomas were expanded and adapted to Hybridomed culture medium (Bichrom AG, Berlin, Germany). The medium was added

Material and Methods

with penicillin/streptomycin 2 mM. The adaptation of the hybridomas was performed gradually to avoid the hybridomas' death. The aminopterin is diluted from the culture by several passages of the cells in hypoxanthine-thymidine (HT, Sigma Aldrich, S. Louise, USA) supplemented medium (approximately 2-3 weeks) before transferring into normal hybridoma growth medium. The passages were from 100% of HAT to 75%, 50%, 25%, 0% of HAT and at the same time from 0% of HT to 25%, 50%, 75% and finally to 100% of HT.

Subsequently, the hybridomas were adapted gradually at the RPMI-1640 20% FCS medium and then they were transferred in Hybridomed, that is a specific hybridoma growth medium. Each time the medium was changed, the hybridomas were analyzed by FACS to confirm the specificity of the same ones against the antigen mesothelin.

FACS (Fluorescence Activated Cell Sorter)

The expression of antibodies anti-Mesothelin was evaluated on different cell lines as follows:

Cells from monolayer cultures were detached with Trypsin-EDTA 0.02%, neutralized with medium of culture and thus harvested. The cells were counted and 2.5×10^5 / 3×10^5 cells were placed in each tube. The cells were washed twice with physiologic solution (NaCl 0.9%) at 4°C and incubated with 100 µl of supernatant of the hybridomas for 1 hour on ice.

After 1 hour of incubation, cells were washed with physiologic solution (NaCl 0.9%) at 4°C and incubated with saturating concentrations (10 µg/ml) of a goat F(ab')₂ anti-mouse immunoglobulin (goat anti-mouse, GAM) fluorescein isothiocyanate (FITC-labeled) (Bechton and Dykinson, Sunnydale, CA, USA) for 20 minutes at 4°C. After

incubation, the cells were washed (once), suspended again in 500 µl PBS and cell associated fluorescence was analyzed by a flow cytometer (FacsCanto, BD Biosciences). The percentage of positive cells and the mean fluorescence intensity (MFI) values were considered in evaluating antigen level of expression.

mRNA EXTRACTION

Total RNA was extracted from the clones that produce the anti-mesothelin antibody. Life Technologies protocol was used as follows:

- 1) *homogenization*: 5×10^6 cells of the anti-mesothelin hybridoma were collected by centrifugation (5 minutes, 150 x g, 5°C) and likewise washed twice in physiologic solution (NaCl 0.9%). Then, the cell pellet was homogenized by resuspension in 1 ml TRizol Reagent (Life Technologies, Gibco BRL).
- 2) *phase separation*: The homogenized sample was incubated for 5 minutes at 15°C to 30°C to allow the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added for 1 ml of TRizol Reagent. The tube was shaken vigorously by hand for 15 seconds and incubated at 15°C to 30°C for 2 to 3 minutes. The sample was centrifuged at no more than 12000 x g for 15 minutes at 2°C to 8°C. Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase whose volume is about 60% of the volume of TRizol Reagent used for homogenization.
- 3) *RNA precipitation*: the aqueous phase was transferred to a fresh tube (you can save the organic phase if isolation of DNA or protein is desired). The RNA was precipitated from the aqueous phase by mixing it with isopropyl alcohol. 0.5 ml

Material and Methods

of isopropyl alcohol for 1 ml of TRizol Reagent used for the initial homogenization was added. The sample was incubated at 15°C to 30°C for 10 minutes and centrifuged at no more than 12000 x g for 10 minutes at 2°C to 8°C. The precipitated RNA, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

- 4) *RNA wash*: the supernatant was removed. The RNA pellet was washed once with 75% ethanol (1 ml 75% ethanol for 1 ml of TRizol Reagent used for initial homogenization). The sample was mixed by vortexing and centrifuged at no more than 7500 x g for 5 minutes at 2°C to 8°C.
- 5) *Redissolving the RNA* : At the end of the procedure, the RNA pellet was briefly dried (air-dry or vacuum-dry for 5-10 minutes). It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. The RNA pellet was dissolved in RNase-free water (25 µl of H₂O DEPC) and was quantified by spectrophotometer (1 unit at OD₂₆₀ corresponds to an RNA concentration of 40µg/ml), reading the absorbance at 260 nm and 280 nm. The isolated RNA has an A_{260/280} ratio (that is the index of purity of the extract) of 1.6 - 1.8 when diluted into distilled water.

RETROTRANSCRIPTION AND cDNA SYNTHESIS

The reaction of retrotranscription required a polymerase named retrotranscriptase that synthesizes a complementary DNA (cDNA) strand from single-stranded RNA. Retrotranscription to cDNA is obtained using SuperScript IITM RT retrotranscriptase (reverse transcriptase kit, invitrogen) following the manufacturer's instructions (Table 5):

Material and Methods

<i>COMPONENTS</i>	<i>VOLUME</i>
Total RNA 5µl	n µl
10 mM dNTP mix	1 µl
Random hexamer (50 ng/µl)	1-5 µl
H ₂ O DEPC	qb 10 µl

The mixture was incubated for 5 minutes at 65°C and then kept on ice. Consequently 9 µl of the following reaction mixture were added (Table 6):

<i>COMPONENTS</i>	<i>VOLUME</i>
10 X RT buffer	2 µl
25 mM MgCl ₂	4 µl
0.1 M DTT	2 µl
RNasi OUT (RNasi inhibitors)	1 µl

The reaction was incubated at 2 minutes at 25°C, after which 1 µl of SuperScript™ II RT was added. Using a thermocycler, the reaction is then incubated for 10 minutes at 25°C, followed by 50 minutes at 42°C and finally 15 minutes at 70°C. The reaction was added with 1 µl of RNasi H and the same one was incubated for 20 minutes at 37°C. RNasi H was added to degrade hybrid RNA strand with DNA that generated during the retrotranscription process.

POLYMERASE CHAIN REACTION (PCR)

The cDNA of the anti-mesothelin hybridoma was amplified by PCR to evaluate its functionality. The amplification was performed with a couple of specific primers (oligonucleotide). The general conditions used for the amplification were (Table 7):

95°C	4 minutes	
95°C	1 minute	30 cycles
56°C	1 minute	
72°C	1 minute	
72°C	7 minutes	
4°C	end cycle	

The following components were used for the reaction (Table 8):

Components	Volume
Primer S15 For 10 pMoli/μl	1 μl
Primer S15 Rev 10 pMoli/μl	1 μl
Gold Buffer 10 X no MgCl ₂	5 μl
MgCl ₂ 25 mM	3 μl
DNTP 10 mM	1.25 μl
Cdna	1-2 μl
Taq Gold 5U/μl	2 μl
H ₂ O	qb 50 μl

The PCR (Polymerase Chain Reaction) product was a fragment of about 350 bp that was analyzed on 2% agarose gel. In order to select the appropriate couple of primers for

Material and Methods

the amplification of fragments coding for the variable regions of heavy and light chains (V_H and V_K , respectively) of the anti-mesothelin hybridoma clone, a panel of 25 *forward* and 4 *reverse primers* was considered for each variable domain (25 V_H forward primers and 4 J_H reverse primers; 25 V_K forward primers and 4 J_K reverse primers). Forward primers were designed considering highly conserved sequences at the 5'-end of DNA fragments for V_H and V_L domains from several families of murine immunoglobulins; reverse primers were instead inferred from J regions located at the 3'-end of V_H and V_L DNA regions. Each forward primer was tested in a PCR reaction that includes a mix of the four reverse primers. Once the best forward primer has been thus selected, it is used in four individual PCR reactions, each one with a single reverse primer. As soon as the appropriate couple of primers has been selected (PCR amplicates were analyzed by agarose gel electrophoresis), the PCR products were sequenced. It was possible to check if the obtained sequences coded for V_H and V_L regions of antibodies, by sequences alignment studies that use BLAST program for bringing into line protein or nucleotide sequences.

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The purpose of the gel is to look the DNA, to quantify it or to isolate a particular band. The gel is constituted by agarose dissolved in TAE 1X (40 mM Tris-Acetate, 1 mM EDTA in deionized H_2O). The DNA is visualized in the gel by addition with ethidium bromide (end concentration of 0.5 $\mu\text{g/ml}$). This binds strongly to DNA by intercalating between the bases and its fluorescent meaning is due to the absorbance of

Material and Methods

invisible UV light and the transmission of energy as visible orange light. The samples of analysis are added with loading buffer 6X that contains:

- 1% SDS, that eliminates DNA-protein interactions, prevents appearance of additional bands due to annealing of DNA molecules with cohesive ends;
- a dye to assess how "fast" your gel is running (0.05% Bromophenol Blue in deionized H₂O);
- a reagent to render your samples denser than the running buffer (so that the samples sink in the well) (50% Glycerol).

Moreover, agarose gel electrophoresis provides the use of reference DNA size marker for the fragment size you expect to see in your sample lanes (the marker used is a mixture of chromatography-purified individual DNA fragments from 0.5 to 10 Kb, New England-Biolabs). The electrophoretic run is conducted to 70-90 Volts.

MONOCLONAL ANTIBODIES PURIFICATION

MAbs were purified from hybridoma supernatants using the affinity chromatography on sepharose-protein G column (resin sepharose-protein G, Pharmacia). The column was earlier equilibrated with bind buffer (PBS 1X ph 7.0).

The column was washed extensively with bind buffer until obtaining a value of absorbance to the spectrophotometer (OD) lower than 0.05.

The antibodies were eluted using glycine 0,1 M ph 3.0 with immediate neutralization into Tris 1 M ph 8.0.

Material and Methods

The fractions containing the antibodies were identified through Comassie staining under denaturing conditions of SDS/PAGE. The fractions having the antibody protein were combined and dialysed against phosphate buffer solution (PBS). Finally, it was concentrated with Amicon Ultra-4 Centrifugal Filter Unit 10000 MWCO (Millipore) and the antibody concentration was determined by spectrophotometric reading of the absorbance at 280 nm (Gill and Von Hippel, 1989). Moreover, the protein concentration was carefully determined by BCA (bicinchoninic acid) Protein Assay.

SDS PAGE (Sodium - Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis)

Polyacrylamide gel electrophoresis (PAGE) of proteins is usually carried out using protein mixture that has been denatured by heating in the presence of an anionic detergent, sodium dodecylsulfate (SDS). SDS is a negatively charged molecule that becomes covalently coupled to proteins along their length upon exposure to heat; apart from denaturing the protein, this imparts also a negative charge in proportion to its length. Upon introduction of the protein sample to the gel and the application of a vertical electric field from the top of the gel to the bottom, proteins are repelled from the negative pole (the cathode) and migrate towards the positive pole (the anode). Due to the molecular sieving effect of the gel matrix, proteins within the mixture become resolved into discrete zone (bands) with the smallest proteins moving furthest through the gel. The proteins will have differentially migrated according to their size; smaller proteins will have travelled farther down the gel, while larger ones will have remained closer to the point of origin. Proteins may therefore be separated roughly according to their size (and thus their molecular weight). It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel, in order to calibrate

Material and Methods

the gel and determine the approximate molecular mass of unknown proteins by comparing the distance travelled relative to the marker. SDS PAGE was performed in *Mini-Protean III Cell* (BioRad) using running buffer 1X and maintaining a constant electric current of 90 Volts.

BLUE COMASSIE STAINING

The gel was soaked for about 20 minutes in a dye solution containing 50% methanol, 10% acetic acid and 0,1% Coomassie Brilliant Blue R250. As the dye has stained the polyacrylamide gel as well as the protein, they needed to destain the gel in order to visualise the protein bands. Polyacrylamide gel was successfully destained using a solution of 30% methanol and 10% acetic acid solution for about 1 hour at room temperature in a stirrer.

BCA (bicinchoninic acid assay)

BCA is a biochemical assay for determining the total level of protein in a solution (0.5 µg/mL to 1.5 mg/mL), similar to Lowry protein assay, Bradford protein assay or Biuret reagent. The total protein concentration is exhibited by a color change of the sample solution from green to purple in proportion to the protein concentration, which can then be measured using colorimetric techniques.

The BCA assay relies primarily on two reactions: First, the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulphate to Cu^{+} (a temperature dependent reaction). The amount of Cu^{2+} reduced is proportional to the amount of protein existing in the solution. Next, two molecules of bicinchoninic acid chelate with each Cu^{+} ion, forming

Material and Methods

a purple-colored product that strongly absorbs light at a wavelength of 562 nm after incubation at 37°C for 20 minutes. The amount of protein presents in a solution can be quantified by measuring the absorption spectra and comparing it with protein solutions with known concentrations.

mAb ANTI-MESOTHELIN/RTA CHEMICAL IMMUNOTOXIN

Anti-mesothelin mAb was derivatized with the heterobifunctional cross-linker SPDP which reacted with its amino groups introducing a dithiopyridylgroup; after derivatization the mAb contained 2.53 sulphidryl group/mAb molecule, on average. Anti-mesothelin-SPDP molecule was conjugated via a reducible disulphide bond to the RTA toxin and the obtained anti-mesothelin/RTA IT was separated from unconjugated RTA by gel filtration chromatography on TSK 3000 SW column; a further purification from unconjugated mAb was achieved by an affinity chromatography passage on Affi Blue gel column.

Fractions that contain the IT, as identified by SDS-PAGE and Western Blot analysis, were concentrated with centricon-10 and dialysed in PBS buffer. The IT concentration was determined evaluating absorbance at 280 nm and confirmed with BCA method.

XTT cell viability assay

To evaluate the IT killing activity dose response assay were performed on mesothelin positive and negative cells growing as a monolayer; the proliferation of IT treated and mock treated cells was measured as orange colored product's absorbance

Material and Methods

XTT Cell Viability Assay provides a simple method for determination of live cell number using standard microplate absorbance readers. Determination of live cell number is often used to assess rate of cell proliferation and to screen cytotoxic agents. XTT is a tetrazolium derivative.

Similar to MTT, XTT measures cell viability based on the activity of mitochondria enzymes in live cells that reduce XTT and are inactivated shortly after cell death. Unlike the water-insoluble formation produced from MTT, XTT is readily reduced to a highly water-soluble orange colored product [*J. Infect. Dis.* **172**, 1153 (1995); *J. Immunol. Methods* **159**, 81 (1993)], thus omitting the solubilization step required for the MTT assay.

The amount of water-soluble product generated from XTT is proportional to the number of living cells in the sample and can be quantified by measuring absorbance at wavelength of 475 nm.

Cells were plated into 96-well tissue culture plates. In general, cells should be seeded at densities between 5,000 and 10,000 cells per well since they will reach optimal population densities within 48 to 72 hours.

The experiment was carried out by adding chemicals or biological agents. The final volume of tissue culture medium in each well should be 0.1mL, and the medium may contain up to 10% Fetal Bovine Serum.

One bottle of XTT Solution and one vial of Activation Reagent were thawed and mixed for two 96-well plates (for one 96-well plate, mix 25 μ L Activation Reagent with 5mL

Material and Methods

XTT Solution to derive activated XTT solution and save the rest of XTT Solution and Activation Reagent for later use).

25 μ L or 50 μ L of the activated XTT solution was added to each well and the plate was incubated in an incubator for 2-24 hours (usually, 2-5 hours is sufficient).

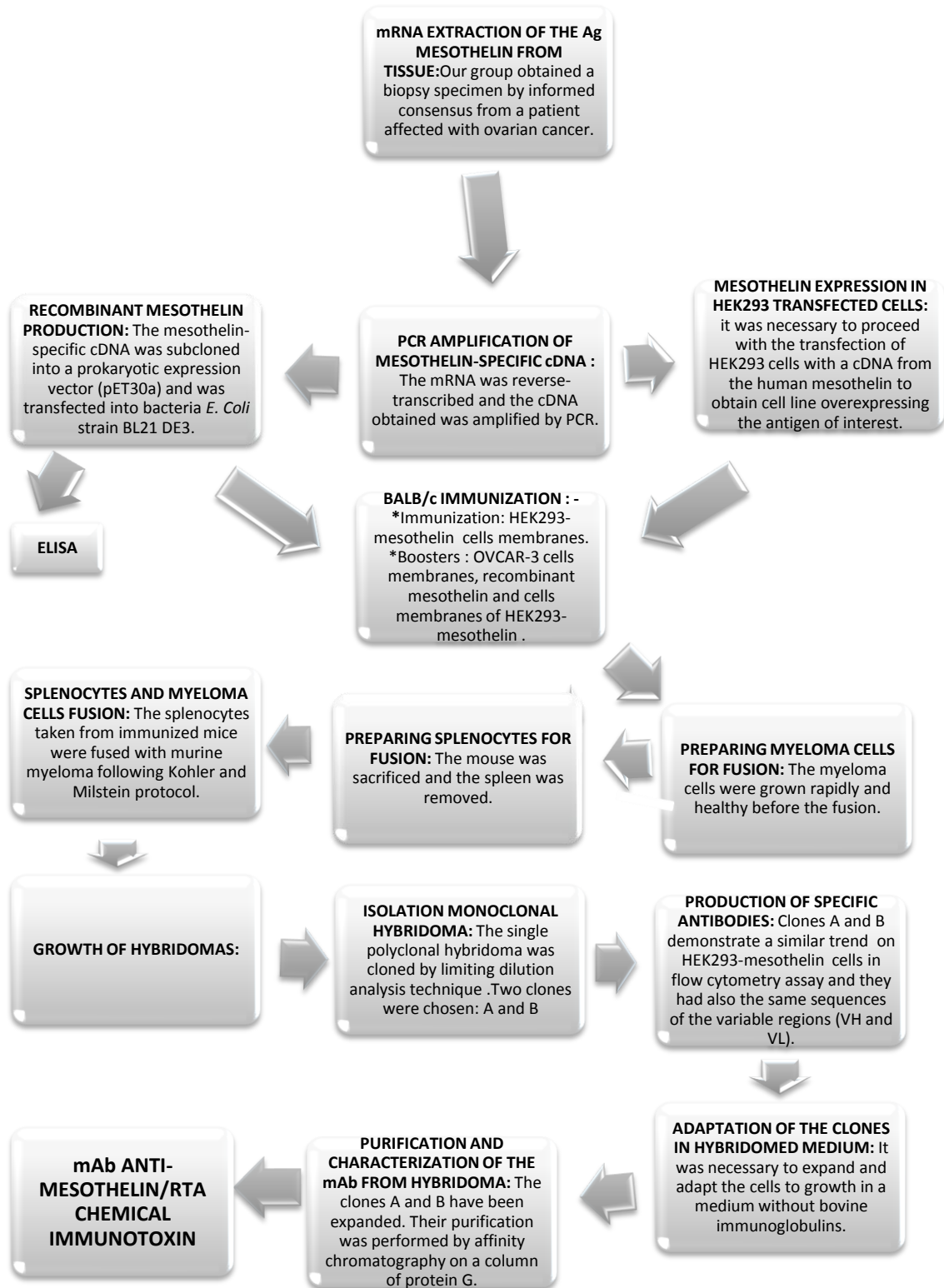
The plate was gently shaken to evenly distribute the dye in the wells.

The absorbance of the samples was measured with a spectrophotometer (ELISA reader) at a wavelength of 450-500 nm. In order to measure reference absorbance (for non-specific readings), use a wavelength of 630-690 nm.

Results are expressed as percent of absorbance (proliferation) respect to mock treated control; to better evaluate the different killing activity we consider IC₅₀, wich is the concentration of IT able to inhibit cellular proliferation of about 50%.

.

Results



RESULTS

TOTAL RNA EXTRACTION FROM AN OVARIAN CANCER TISSUE

Mesothelin was cloned from a surgical biopsy specimen of a women suffering from ovarian cancer (tissue was obtained with informed consensus). This sample was processed according to the TRIzol Reagent protocol, based on the separation of nucleic acids RNA and DNA and the proteins. The aqueous phase containing total RNA was washed, precipitated with 75% ethanol to obtain an total RNA pellet and subsequently resuspended in DEPC water. The quality and concentration of RNA were evaluated by a spectrophotometer measuring the absorbance ratio 260/280 nm.

RT-PCR AND PCR AMPLIFICATION OF MESOTHELIN-SPECIFIC cDNA

Total RNA was reverse-transcribed using random hexamer primers and the obtained cDNA was amplified by PCR using specific primers allowing amplification and cloning of mesothelin cDNA. The amplification reaction was subjected to agarose gel electrophoresis (Figure 13) which permitted us the identification of the amplified mesothelin cDNA according to its size (approximately 1,900 bp).

Results

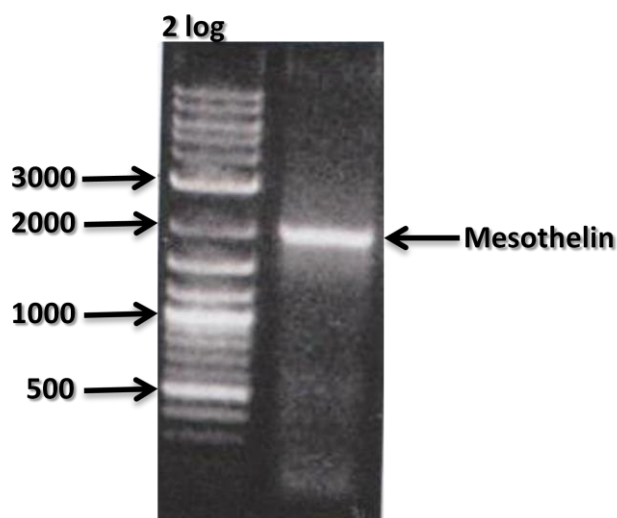


Fig. 13 The band obtained by PCR amplification of the mesothelin cDNA. The agarose gel run shows that the PCR product is about 1,900 bp.

RECOMBINANT MESOTHELIN PRODUCTION

The mesothelin cDNA was subcloned into a prokaryotic expression vector (pET30a) and then transfected into bacteria *E. Coli* strain BL21 DE3. After induction overnight, bacteria were diluted in fresh medium and grew in 1 L of LB medium with kanamycin until a spectrophotometric absorbance of OD 0.8 at 600 nm. Then they were induced with IPTG for 3 hours at 30° C. The bacterial cells were afterwards lysed in buffer IB containing lysozyme, DNase I, PMSF and complete protease inhibitor (Roche); to improve bacteria lysis the mixture was subjected to repeated sonication cycles. The sonication process provides mechanical breaking of the cells whereas the buffer IB acts osmotic lysis of the membrane; the presence of protease inhibitors preserves our recombinant mesothelin from degradation. After the centrifugation of the lysis product and the elimination of the supernatant, we proceeded with the solubilization of the inclusion bodies pellet to obtain mesothelin. In previous experiments we had confirmed

Results

that the recombinant Mesothelin was accumulated during bacterial growth in the inclusion bodies.

In order to create new anti-mesothelin antibodies recognizing the native isoform of this human tumor antigen for immunotherapeutic purposes, it was necessary to develop a refolding protocol. The refolding process was performed by sequential dialysis steps in different buffer solutions. We eliminated progressively the sodium lauryl sulfate (denaturant reagent) and then DTT reagent in order to slowly refold the protein. The dialysis protocol that has proven to show a very low level of aggregation products, took advantage from a series of buffers based on Tris-HCl; the last buffer provided the addition of arginine and glutathione (reduced and oxidized). Glutathione performed the refolding of the protein, whereas arginine protected it from potential reaggregation. The identification of Mesothelin produced in our bacterial system was carried out by western blot (WB) developed with the commercial anti-mesothelin mAb K1 (Figure 14). The protein purification was performed by affinity chromatography using the affinity of imidazole rings of recombinant mesothelin poly-histidine tail for Ni-NTA resin. Once the chromatographic column was loaded with the refolded protein solution and some washing steps were performed, Mesothelin was eluted with a solution containing a high concentration of imidazole, which competes with the protein molecules for the binding to the resin. Elution fractions were analyzed by spectrophotometric reading and run on polyacrylamide gels in order to determine the protein concentration and its purity. Recombinant mesothelin was used for mice immunizations and ELISA assays.

Results

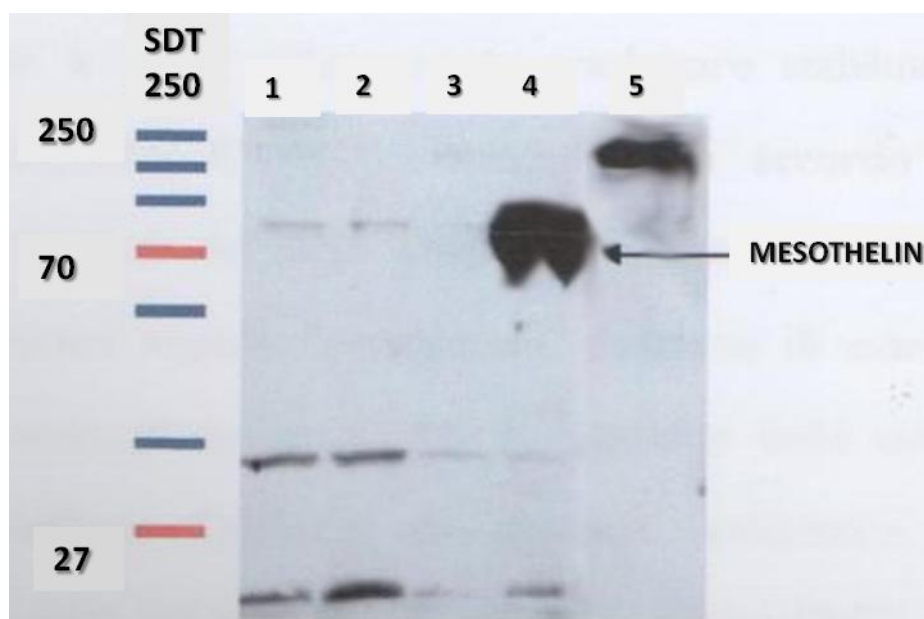


Fig. 14 Western blot with anti-mesothelin mAb K1 of BL21 DE3 bacteria lysate. 1) not induced cytoplasm 2) induced cytoplasm 3) not induced inclusion bodies 4) induced inclusion bodies 5) lysate of ovarian carcinoma (positive control).

MESOTHELIN TRANSFECTED HUMAN HEK293 CELLS

Above all, it was necessary to proceed with the transfection of human HEK293 cells with mesothelin cDNA to obtain a cell line overexpressing the antigen of interest.

This new cell line was used for both immunization protocols and functional characterization of the synthesized mAbs by flow cytometry.

HEK293 cells were stably transfected with the plasmid pcDNA3.1-mesothelin, previously linearized. The transfection was performed with Lipofectamine 2000 following manufacturer's protocol (Invitrogen).

pcDNA3.1 vector contains neomycin resistance gene. The selection and maintenance of the transfected cells were performed by using antibiotic G418 that only permits the survival of transfected cells with neomycin resistance genes.

Results

Mesothelin expression on the surface of the transfected HEK293 cells was confirmed by flow cytometry using a commercially available anti-mesothelin mAb K1.

FACS analysis was performed on HEK293-mesothelin cells and HEK293 wt cells as negative control.

The transfection efficiency was analyzed considering two parameters:

Mean Fluorescence Intensity (MFI) and the percentage of cells depicted in the channel select as positive..

As summarized in Table 9, mAb K1 showed a MFI value of 8,650 on HEK293-mesothelin cells compared to a MFI value of 152 on HEK293 wt cells. Irrelevant MFI values were detected with isotype matched control Ab on the same cells (MFI 216 and MFI 213 respectively).

The positive control Ab anti MHC-1 B912.1 showed an MFI value of 2,096 on HEK293-mesothelin cells and MFI value of 1,178 on HEK293 wt cells; Figure 15 confirms that mAb K1 strongly stained HEK293-mesothelin cells whereas irrelevant staining was detected on HEK293 wt cells.

Results

	<i>HEK293-mesothelin</i>		<i>HEK293 wt</i>	
<i>Sample</i>	MFI	% of positive cells	MFI	% of positive cells
Negative control	216	2.4	213	0.7
Positive control	2,096	93.4	1,178	85.2
Ab K1	8,650	88.3	152	1.4

Table 9 Data obtained by flow cytometer analysis performed on HEK293-mesothelin and HEK293 wt cell lines using anti-mesothelin mAb K1 compared to isotype matched Ig negative control (Ab Goat Anti Mouse – FITC - Ab GAM-FITC) and positive control (Ab anti MHC-1 B912.1).

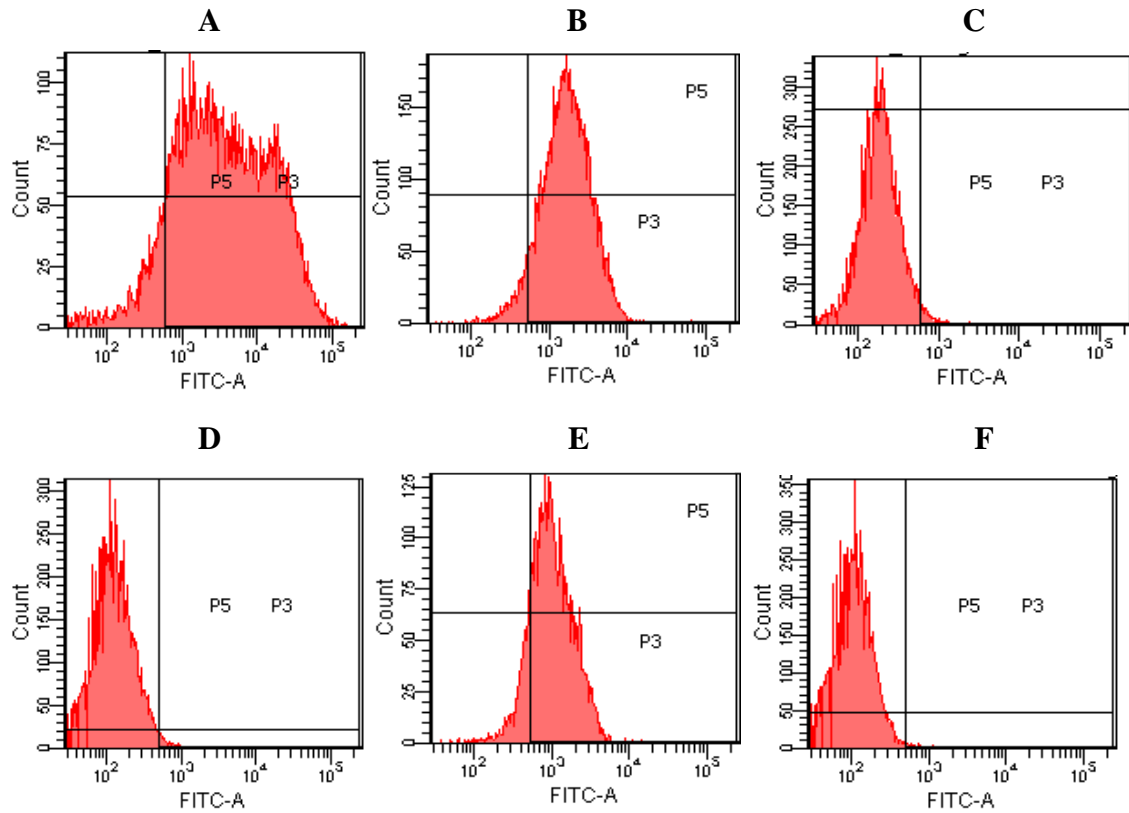


Fig. 15 FACS analysis of the HEK293-mesothelin cells (A, B, C) and HEK293 wt cells (D, E, F). **A, D)** mAb K1; **B, E)** Ab anti MHC-1 B912.1; **C, F)** Ab GAM-FITC.

BALB/c IMMUNIZATION WITH MESOTHELIN EXPRESSING CELL MEMBRANES

BALB/c mice were immunized as described in chapter “material and method”. The anti-mesothelin Ab responses were primarily monitored in mice immunized sera by flow cytometry analysis on HEK293-mesothelin and HEK293 wt cell lines. Serum of non-immunized mice represents the negative control. As shown in Table 10, MFI value of one out of three serum of immunized mice was 14,779 on HEK293-mesothelin cells compared to a MFI value of 703 on HEK293 wt cells. Moreover, MFI value of the non-immunized mouse serum shows nearly the same MFI value on both HEK293-mesothelin and on HEK293 wt cells (MFI of about 183). The immunized mouse, whose serum showed the highest anti mesothelin Ab titer, received a booster immunization by an intraperitoneal injection of HEK293-mesothelin cell membranes and an intravenous injection of the mesothelin recombinant protein, at days -3, -2, -1 from the splenectomy. Figure 16 confirms that immunized mouse serum strongly stained HEK293-mesothelin cells, whereas poor staining was detected on HEK293 wt cells; it is important to remind that antiserum generated in this way also contained considerable amounts of other antibodies directed against a variety of membrane antigenic determinants (this antiserum was said to be polyclonal).

Results

Sample	MFI		MFI	
	HEK293-mesothelin		HEK293 wt	
	Dilutions		Dilutions	
	1:200	1:1000	1:200	1:1000
not immunized mouse serum	183	120	147	131
immunized mouse serum	14,779	4,322	703	249

Table 10 Mean Fluorescence Intensity (MFI) values obtained by flow cytometry analyzing an immunized mouse serum and a non-immunized mouse serum on both HEK293-mesothelin and HEK293 wt cells.

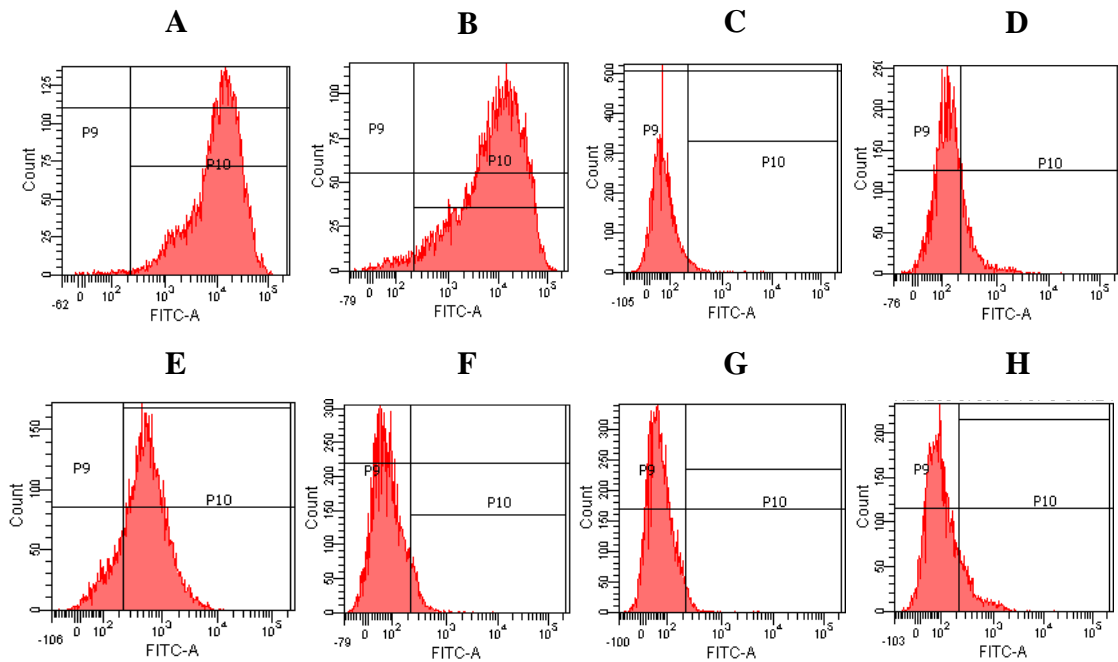


Fig. FACS analysis performed on HEK293-mesothelin cells (A, B, C, D) and HEK293 wt cells (E, F, G, H). **A, E)** immunized mouse serum; **B, F)** mAb K1; **C, G)** Ab GAM-FITC; **D, H)** non-immunized mouse serum.

Results

Based on these data, Hybridomas were prepared by the technique of hybridization of an immortal myeloma cell line with an antibody-producing B cell, as developed by Kohler and Milstein in the late 1970s.

GENERATION AND GROWTH OF HYBRIDOMAS

The splenocytes taken from an immunized mouse spleen were fused with a murine myeloma and the cell suspension obtained was then seeded in two 24 wells-plates. During the two weeks following the fusion, hybridoma cell growth was monitored by evaluation with a microscope. The supernatants of polyclonal hybridomas were analyzed by flow cytometry to detect the presence of antibodies recognizing the antigen of interest. Assays were performed on antigen positive cells (HEK293-mesothelin) and antigen-negative cells (HEK293 wt) in order to confirm the specificity of the recognition. Only one out of the 48 analyzed hybridoma supernatants stained mesothelin positive cells in flow cytometry whereas irrelevant staining was detected for the other hybridomas (Figure 17). Supernatants were also analyzed by ELISA (Enzyme Linked Immunosorbent Assay); all clones recognized recombinant mesothelin because, we suppose, it was more immunogenic than the native form of the protein (data not shown).

Results

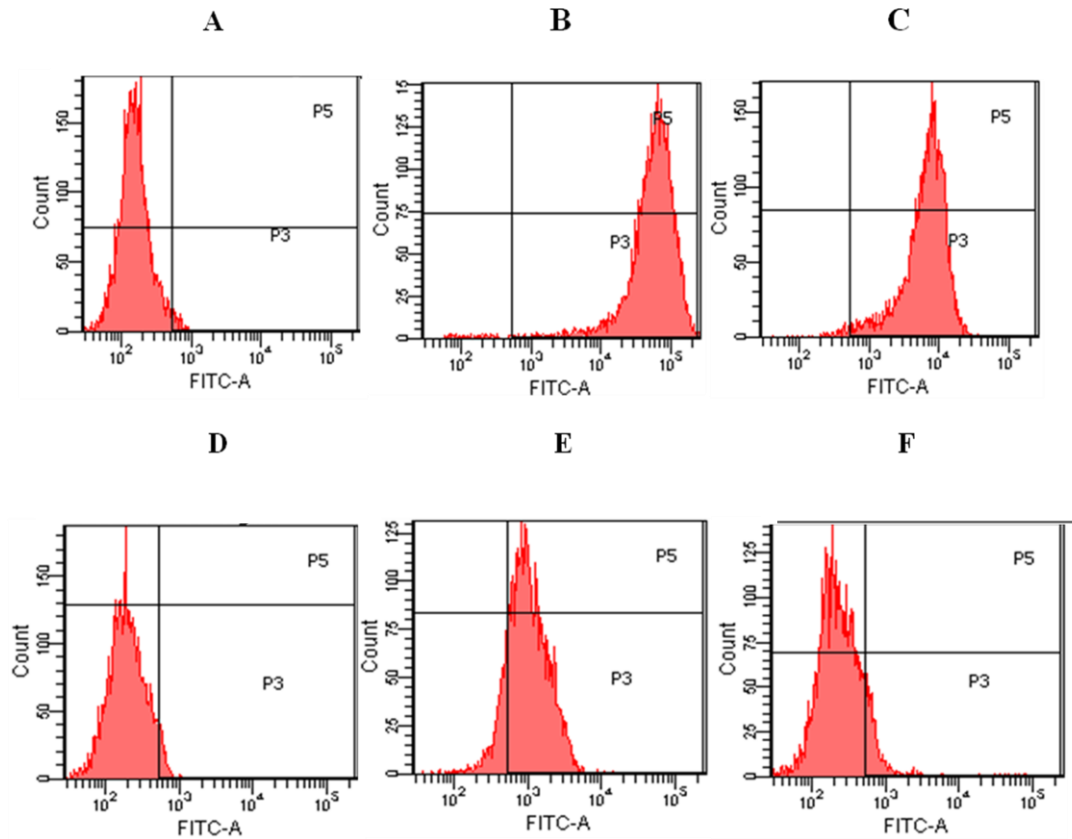


Fig. 17 FACS analysis carried out on HEK293-mesothelin cells (A, B, C) and on HEK293 wt cells (D, E, F) to 94ssess superantants of polyclonal hybridomas. The negative control is represented by Ab goat anti-mouse (A, D) and the positive control is represented by Ab anti MHC-1 B912.1 (B, E); supernatants of the polyclonal hybridoma of interest (C, F).

The positive polyclonal hybridoma supernatant (Figure 17) was analyzed in flow cytometry more than one time with intervals of about 8 days to verify that the antibody production and the specificity were stable over time (Table 11).

Results

HEK293-mesothelin cells	MFI FITC	% of positive cells
Negative control	176	2.2
First analysis	50,876	94.6
Second analysis	38,991	93.2
Third analysis	73,915	98.0

Table 11 Values obtained by flow cytometric analysis of the single mesothelin positive polyclonal hybridoma assessed at intervals of 8 days on HEK293-mesothelin cells.

The variability of the MFI between the second control and the third one is due to different Ag expression in transfected cells because the same behavior was also observed using the monoclonal Ab K1.

The table shows that the hybridoma did not lose its ability to produce specific antibodies.

FROM POLYCLONAL TO MONOCLONAL HYBRIDOMA

The positive polyclonal hybridoma was then cloned by limiting dilution assay (LDA) in three 96-wells plates. During growth, some wells that showed to have more than one clone/well were removed. The individual clones were then expanded and the supernatant was analyzed in flow cytometry to assess the ability of cells to produce a specific monoclonal antibody recognizing the antigen of interest.

Results

FACS analysis identified 34 positive clones. The assays were performed more than one time, on HEK293-mesothelin cells and HEK293 wt cells to check the stability of the antibody production by the clones themselves. Two clones were chosen: A and B (Figure 18),

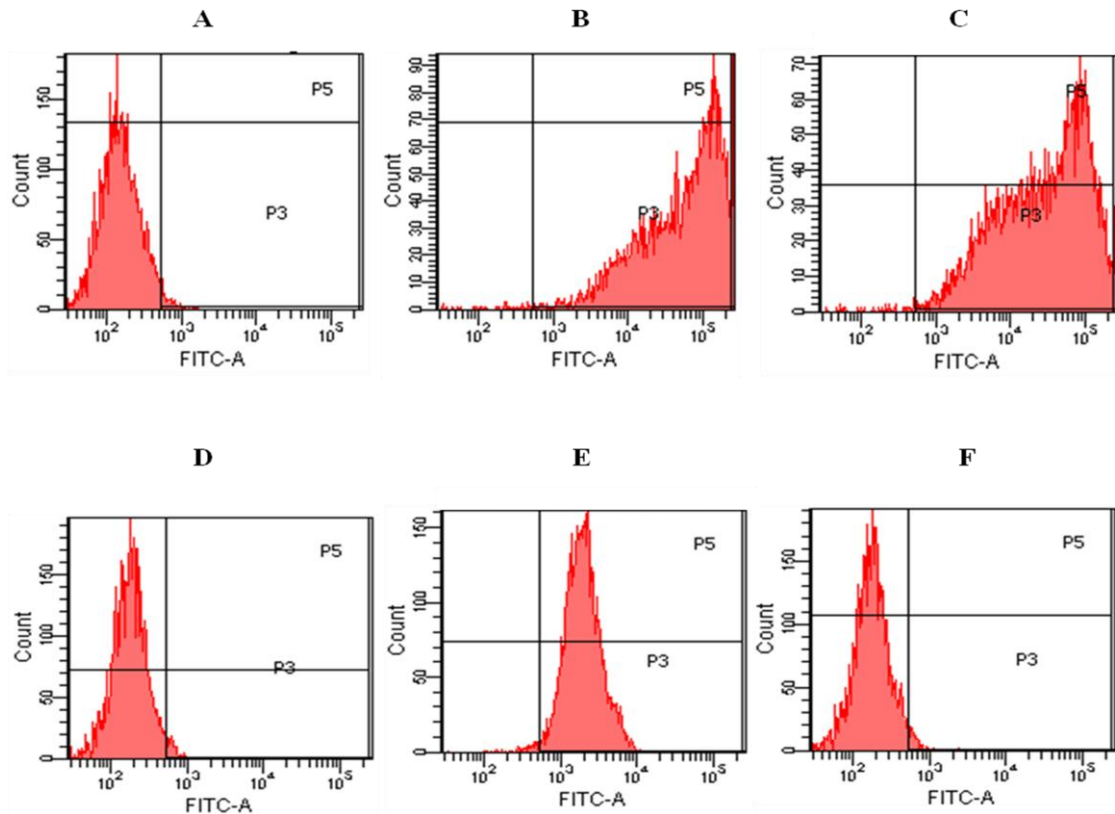


Fig. 18 FACS analysis of the clone A on HEK293-mesothelin cells (A, B, C) and HEK293 wt cells (D, E, F). Ab GAM-FITC was used as a negative control (A, D); Ab anti MHC-1 B912.1 (B, E) was used as a positive control to compare the binding properties of the clone A (C, F). The clone B showed a staining superimposable to that of clone A (data not shown).

As summarized in Table 4, comparing MFI values of the supernatants with positive control MFI value, it appears that the reactivity on HEK293-mesothelin transfected cells is specific. By the contrast, irrelevant MFI value (comparable to negative control) was detected on HEK293 wt, thus indicating that the supernatants did not show non specific recognition (Table 12).

Results

HEK293-mesothelin cells	MFI	% of positive cells
Negative control	200	2.0
Positive control	56,481	95.8
Clone A	36,599	92.5
Clone B	47,176	91.3

Table 12 FACS analysis to evaluate anti-mesothelin antibodies produced by clone A and clone B supernatants. Data show MFI values and % of positive cells obtained on HEK293-mesothelin cells compared to MFI values obtained with the negative control (Ab GAM-FITC) and the positive control (mAb K1).

HEK293 wt cells	MFI	% of positive cells
Negative control	202	2,7
Positive control	2,252	98.3
Clone A	212	3.0
Clone B	201	2.4

Table 12 MFI values and % of positive cells obtained by flow cytometer analysis of clone A and clone B hybridoma supernatants on HEK293 wt cells. Data indicate that the supernatants contain antibodies that explicitly do not recognize specific antigen negative cells. Negative control, Ab GAM-FITC. Positive control, Ab anti MHC-1 B912.1.

Results

The supernatants of these clones were further analyzed on cells constitutively expressing the mesothelin (OVCAR-3 cell line) (Table 13); this analysis was important to assess whether we produced Abs recognizing the constitutive form (or native form) of the antigen. Mesothelin is expressed with different glycosylation grade and refolding processes on tumor cells; for this reason, it's important to evaluate the ability of our Ab to recognize these modified form. Moreover, the clones were also assessed on different antigen negative cell lines to better evaluate the non specific binding, excluding the phenomena of cross-reactivity. Flow cytometry analysis were carried out on:

- Mesothelin expressing cells OVCAR-3;
- Mesothelin not expressing cells: C6, U937, A431, PC3, Jurkat;

From these additional studies we have confirmed the ability of these antibodies to recognize the constitutive form (or native form) of the antigen and the specificity of the antibodies themselves.

Results

The data were compared with the positive control mAb anti-mesothelin K1.

OVCAR-3 cells	MFI	% of positive cells
Negative control	246	6.4
Positive control	3,259	90.2
Clone A	1,430	72.4
Clone B	2,695	88.1

Table 13 FACS analysis to evaluate anti-mesothelin antibodies produced by clone A and clone B. Data show MFI values and % of positive cells obtained on OVCAR-3 cells.

As shown in Table 6 the antibodies produced by clones A and B are able to recognize the antigen mesothelin expressed on the membranes of OVCAR-3 cells. The results shown in Table 14 summarize experiments performed on antigen negative cells; the MFI values obtained by clone A and clone B supernatants are comparable to MFI value of the negative control, demonstrating that no aspecific staining is detectable on antigen negative cells.

Results

CELL LINE	U937		Jurkat		A431		C6		PC3	
SAMPLE	MFI	<i>% of positive cells</i>	MFI	<i>% of positive cells</i>	MFI	<i>% of positive cells</i>	MFI	<i>% of positive cells</i>	MFI	<i>% of positive cells</i>
NEGATIVE CONTROL	149	2.1	554	1.1	227	5.2	156	2.7	228	2.5
CLONE A	168	2.2	152	0.4	245	7.0	182	3.7	247	4.8
CLONE B	165	2.6	160	0.4	247	7.3	193	4.7	259	5.8

Table 14 FACS analysis to evaluate anti-mesothelin antibodies produced by clone A and clone B. Data show MFI values and % of positive cells obtained on different antigen (mesothelin) negative cell lines compared to MFI values obtained with the negative control.

Taken together these results suggest that antibodies produced by clones A and B are able to recognize the antigen mesothelin in the native and recombinant form.

GROWTH OF THE CLONES IN HYBRIDOMED MEDIUM

It was necessary to expand and adapt the cells to growth in a medium without FCS and therefore without bovine immunoglobulins: Hybridomed Medium; this allows to purify the antibody without contamination by other Abs of bovine origin. The adaptation has been conducted gradually moving from a HAT medium 20% FCS to a HT medium 20% FCS and then RPMI 20% FCS until to RPMI 10% FCS and finally to the medium Hybridomed.

Results

During the adaptation phase, the secretion and the functionality of the produced Abs were always monitored by flow cytometry.

PURIFICATION AND CHARACTERIZATION OF THE NEW mAbs

The clones A and B that produce the anti-mesothelin antibodies have been expanded to obtain a sufficient amount for purification. Their purification from the hybridoma growth medium was performed by affinity chromatography on a protein G column. Since the two clones A and B produced the same Ab, we wanted to investigate whether one of the two clones was more productive than the other one.

The purified antibodies from the two clones were quantified by photometric absorbance at 280 nm and by BCA assay and the results obtained were comparable. From 250 ml of culture medium of each of the two clones 3mg of anti-mesothelin Ab were approximately obtained. From now, we will only consider data concerning mAbs produced by clone A hybridoma because, after sequencing VH and Vk regions, we disclosed an absolute identity among this two mAbs (A and B). The purity of the antibody was then analyzed by SDS-PAGE (Figure 19).

Results

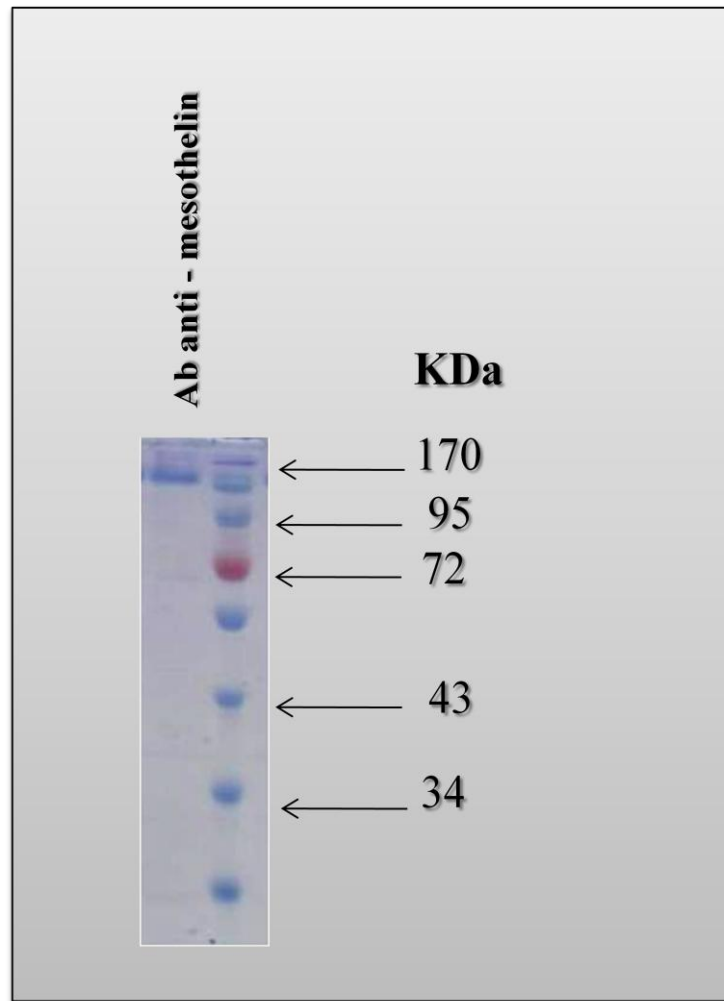


Fig. 19 10% polyacrylamide gel and Coomassie staining of the Ab anti-mesothelin obtained by affinity column purification.

Polyacrylamide gel shows a single band at about 150 kDa, demonstrating the purity of our preparation.

The purified antibody was then analyzed in flow cytometry to evaluate if it maintained the ability to bind specifically its target antigen. The analysis was performed on HEK293 wt, HEK293-mesothelin (data not shown) and OVCAR-3 cell lines.

As shown in Table 8, the Ab maintains the ability to recognize specifically the antigen.

Results

OVCAR-3 cells	MFI	% of positive cells
Negative control	148	0.6
Positive control	56,575	97.9
Clone A purified	38,135	98.5

Table 15: MFI values and % of positive cells obtained by flow cytometric analysis conducted to test the specificity of recognition of the anti-mesothelin mAb on OVCAR-3 cells after the purification.

The Ab was then characterized by assessing the affinity by binding studies on HEK293-mesothelin cells. Scalar concentrations of the Ab of interest and Ab K1 were used for the assay. Subsequently, the samples were incubated with secondary antibody Goat Anti Mouse FITC at a saturating concentration and analyzed by flow cytometry.

Figure 20 shows that decreased concentrations of the Ab in object correspond to decreased binding percentage. The picture describes the results of the cytofluorimetric analysis, where MFI values were revised according to the maximum MFI value which was assigned to the 100% saturation of antigenic sites (mesothelin present on the cell membrane of HEK293-mesothelin).

The results are described by a sigmoid curve that allows the comparison of the two antibodies binding capacity. Our “home-made “ anti-mesothelin mAb has better affinity compared to the commercial one K1. Our “home-made” anti-mesothelin mAb saturates at a concentration of about 9 nM, while mAb K1 requires slightly higher concentrations (18 nM).

Results

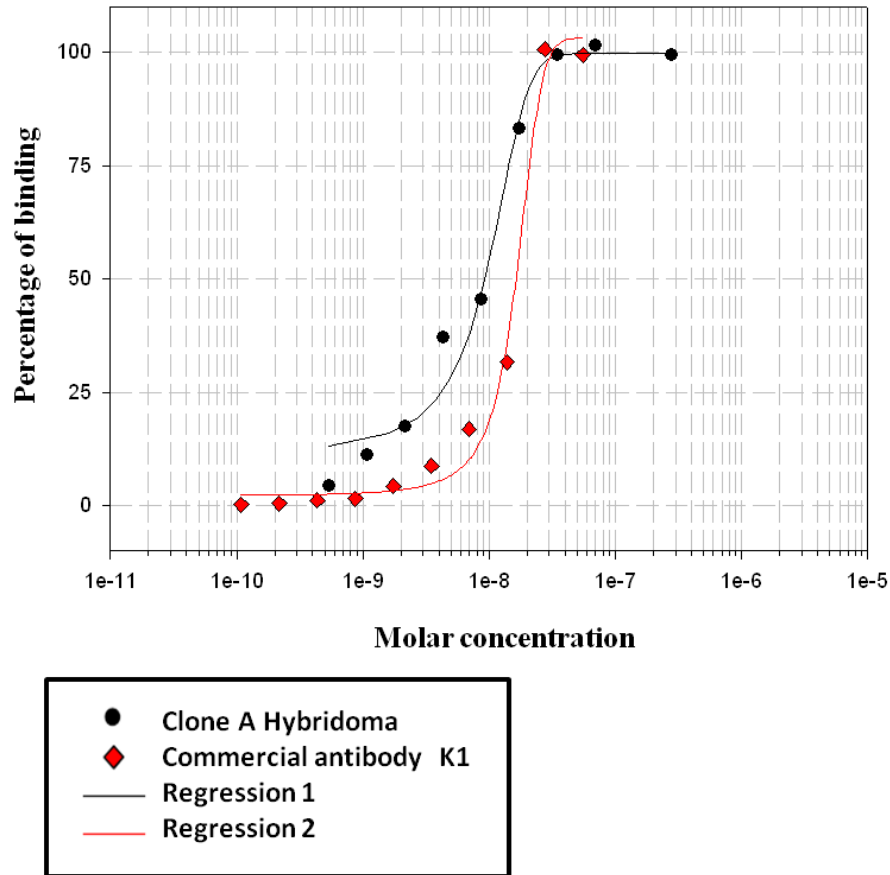


Fig. 20 Binding curves. FACS analysis about the saturation of mesothelin existing on the membrane of HEK293 transfected cells in the presence of scalar concentrations of Ab K1 and “home-made” anti mesothelin mAb produced by hybridoma technology. Data revised according to the maximum MFI value which was assigned to the 100% saturation of antigenic sites.

According to the results showing high specificity and high affinity, we decided to identify the sequences of the antibody variable regions (VH and VL regions) to create antibody fragments (scFv format) from the whole anti-mesothelin mAb. Total RNA was purified from hybridoma cells secreting monoclonal antibodies and VH and VL regions were obtained by reverse transcription. Since mouse Ig employs preferentially κ light chains (1:10 ratio λ/κ), we used for VL amplification degenerated primers for V κ and J κ domains only. Primers for this first amplification were designed to match the leader regions of the VH and V κ chains and both constant regions of together heavy and light

Results

chains. 25 PCR reactions were carried out for each V chain combining 25 different VH or Vk murine domain forward primers with a mix of four JH or Jk corresponding reverse ones as showed in Figure 10. The products of VH and Vk amplification can be visualized on a 2% agarose gel and are nearly 350 bp long; 7 out of 25 primers amplify the Vk and 13 out of 25 the VH; positive controls were obtained by Vk and VH sequences amplification of an anti-human CD3 murine hybridoma; in the picture above we can observe that because of the primer sequences degeneration, we have amplification products from several V-primers. Among them, according to band intensity and low amplification background, we have chosen one VH (lane 28) and one Vk (lane 3) forward primers.

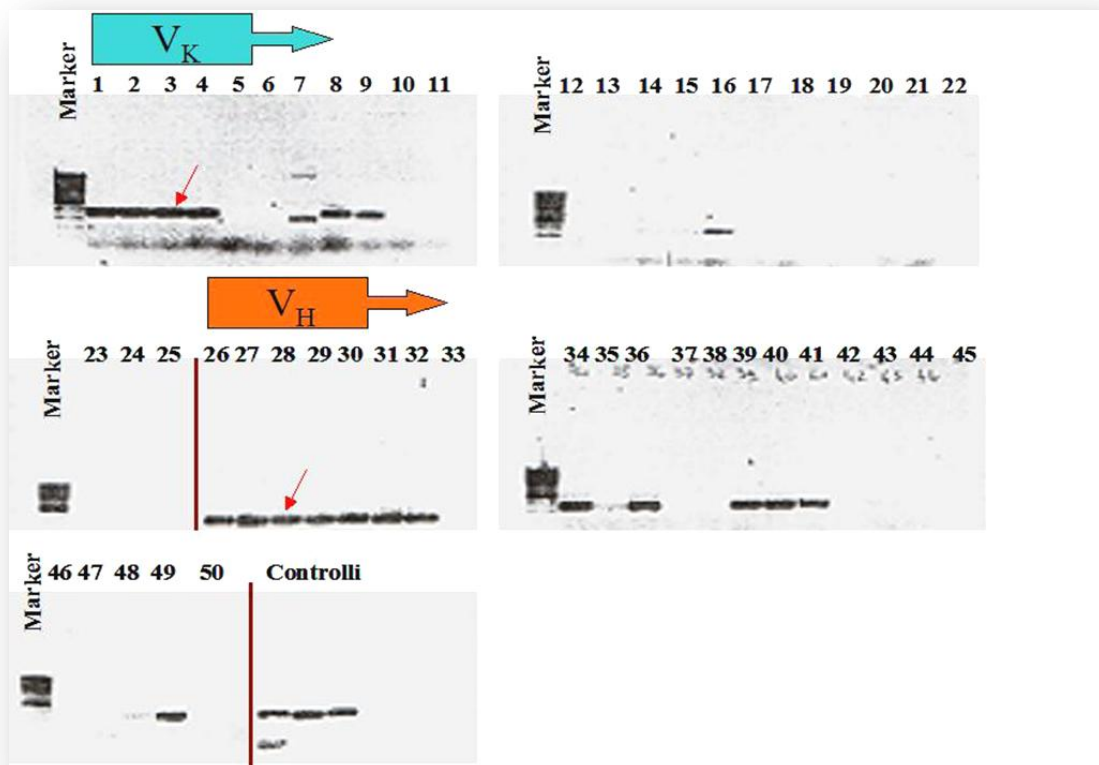


Fig. 21 PCR products obtained by PCR amplification of the anti-mesothelin hybridoma cDNA with 25 forward primers for Vk region (blue) and 25 forward primers for VH region (red).

Results

At this point, it was important to test the “joining” (J) primers separately in order to identify JH and Jk ones leading to the best matching pair for heavy and light chain amplifications. V genes obtained by PCR were sequenced using the same forward and reverse primers as for PCR reactions. The sequences of the variable regions of heavy and light chains of anti-mesothelin Abs produced by the two hybridomas are shown below. The Complementary Determining Region [CDRs (CDR1, CDR2 and CDR3)] represented by underlined regions have been edited because of patent restriction.

The sequence of the variable heavy chain VH of the mAb clone A is the following:

ELEGPELSCVASGFTFXXXXXXXXWVRQTPEKRLEWVXXXXXXXXXXXXXXXXXXXX
XRFTISRDNARNILFLQMSSLRSEDTAMFYCARXXXXXXXXXXXXWGAAGTTVT
XLLK.

The sequence of the variable light chain of the mAb clone A Vk is the following:

LLGQRATISYXXXXXXXXXXXXXXXXXXXXWNQQKPPGQPPRLLIYXXXXXXXXGVPA
RFSGSGSGTDFTLLNIHPVEEEDAATYYCXXXXXXXXXXEGGPSWK.

The sequences of the variable regions of mAb clone B were the same of mAb clone A.

Based on a comparison method of the sequences described by Kabat and Wu (1991), it was evident that VH and Vk aminoacidic sequences of our “home-made” mAb anti-mesothelin corresponded to the VH and Vk aminoacidic sequences of “standard” antibody.

Results

To confirm this, clone A shown the aminoacidic sequence **ASGFTF** before of the heavy chain CDR1 region, whereas the aminoacidic sequence **CAR** was before of the CDR3 region.

Moreover, the aminoacidic sequence **WNQQKP** after the light chain CDR1 region and the aminoacidic sequence **DAATYYC** before of the CDR3 region described this correspondence.

Taken together, these results suggest that mAbs produced from the clone A and B are identical, because they are characterized by the same variable light and heavy chains. The evidence of this similarity between antibodies produced by clone A and clone B comes from the complementarity-determining regions CDR1, CDR2, CDR3 of these mAbs that are able to bind the same epitope and with the same affinity.

SYNTHESIS OF THE ANTI MESOTHELIN IT: mAb –spdp-nRTA

The whole mAb was chemically linked to ricin A chain toxin (RTA) in order to create an IT anti Mesothelin: mAb–spdp-nRTA. The IT killing efficacy will be studied on mesothelin positive and mesothelin negative cells. Chemical linked IT was synthesized by conjugation of RTA toxin to the mAb anti mesothelin via a disulfide bond, using the cross-linker SPDP molecule. After reducing cysteines on RTA protein by DTT and the derivatization of the mAb with SPDP, the binding reaction of these two reagents took place for about 2 days at 4°C; the obtained IT was then purified from unconjugated reagents by a first step of gel filtration chromatography. The chromatogram in Figure 8 displays a purification run obtained through a Bio-Rad FPLC apparatus. A second chromatographic purification on an Affi-Blue gel column allowed us to remove the

Results

unconjugated mAbs. It is mandatory to eliminate these free mAbs, because they are able to compete with IT for the binding to the target antigen; this competition could mask the real killing efficacy of the IT in the cells assay (Figure 22).

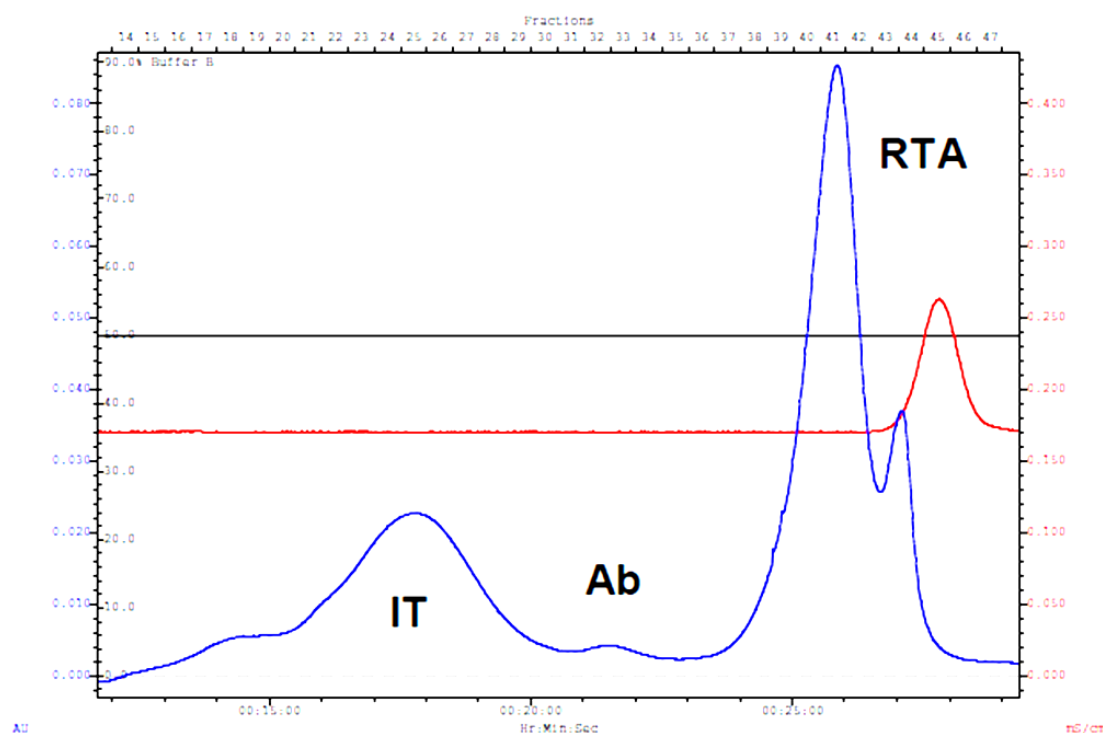


Fig. 22 Chromatogram of a purification run of the IT on gel filtration column.

“IN VITRO” IMMUNOTOXIN EFFICACY

In order to evaluate the cytotoxic effect of anti-mesothelin ITs, we measured the proliferation of mesothelin positive and mesothelin negative cells once treated in a dose-response assay with ITs and toxin alone. Cytotoxic effects were compared in terms of IC_{50} , which defines the reagent concentration able to inhibit cell proliferation of about 50% respect with a mock treated control. Figure 23 describes the data obtained from a typical experiment performed on mesothelin positive cells.

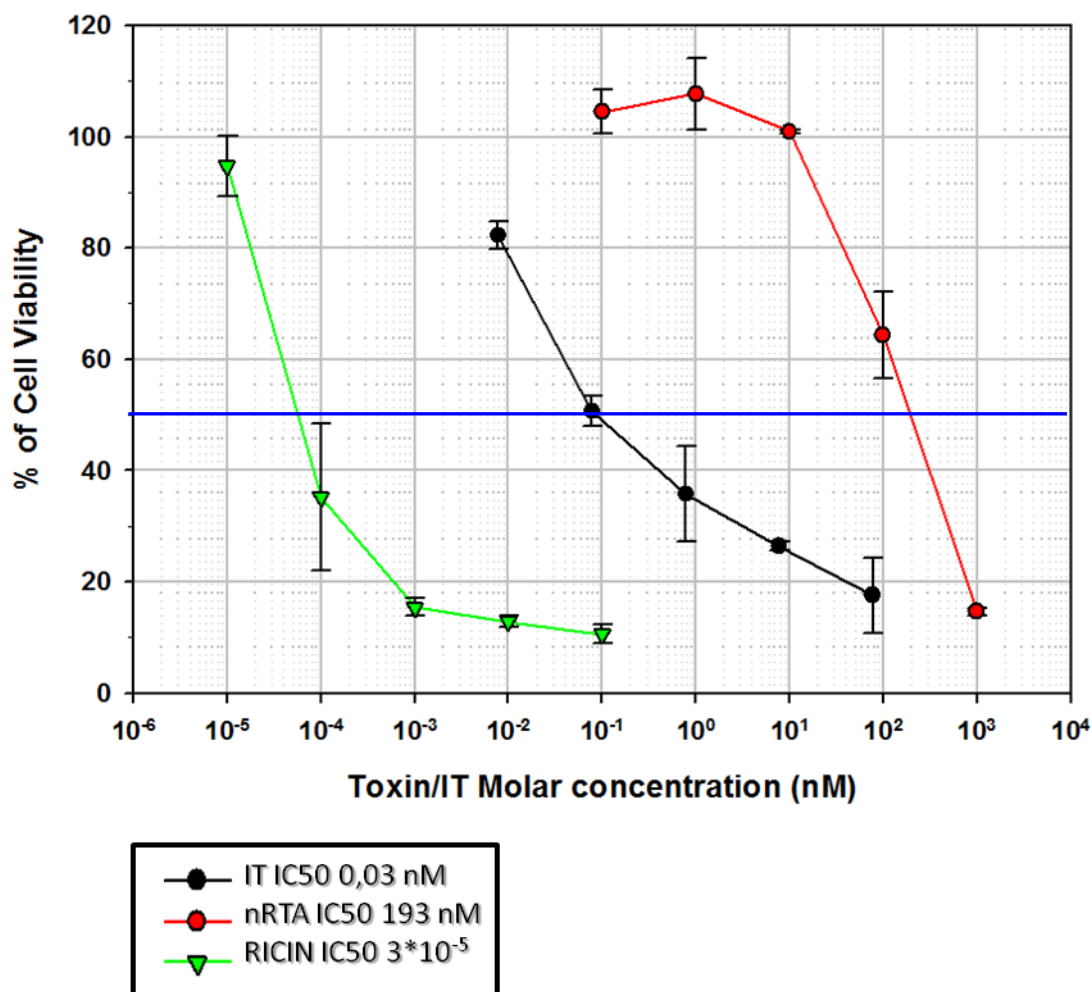


Fig. 23 Dose-response curves obtained treating HEK293-mesothelin cells with increasing concentration of molecules described in the picture. The cytotoxic efficacy, XTT assay, was measured considering the absorbance of water-soluble orange colored product after 36 hrs. Samples: IT – immunotoxin; nRTA,; RICIN.

As illustrated in the Figure 23, chemical IT (anti-mesothelin mAb-spdp-nRTA) showed an high cytotoxic activity (0.03 nM) on HEK293-mesothelin cells after 36 hrs of incubation; the nRTA toxin used for the synthesis of the IT showed an IC_{50} of about 193 nM on the same cells. As expected ricin alone showed the highest cytotoxic activity.

The specificity of IT mediated killing was further investigated by comparing the cytotoxic effects on HEK293 wt cells after 36 hrs of incubation.

Results

As illustrated in the Figures 24, chemical IT (anti-mesothelin mAb-spdp-nRTA) did not show cytotoxic activity on above-mentioned cells. In the same manner Ricin alone confirmed the highest cytotoxic activity on these cells.

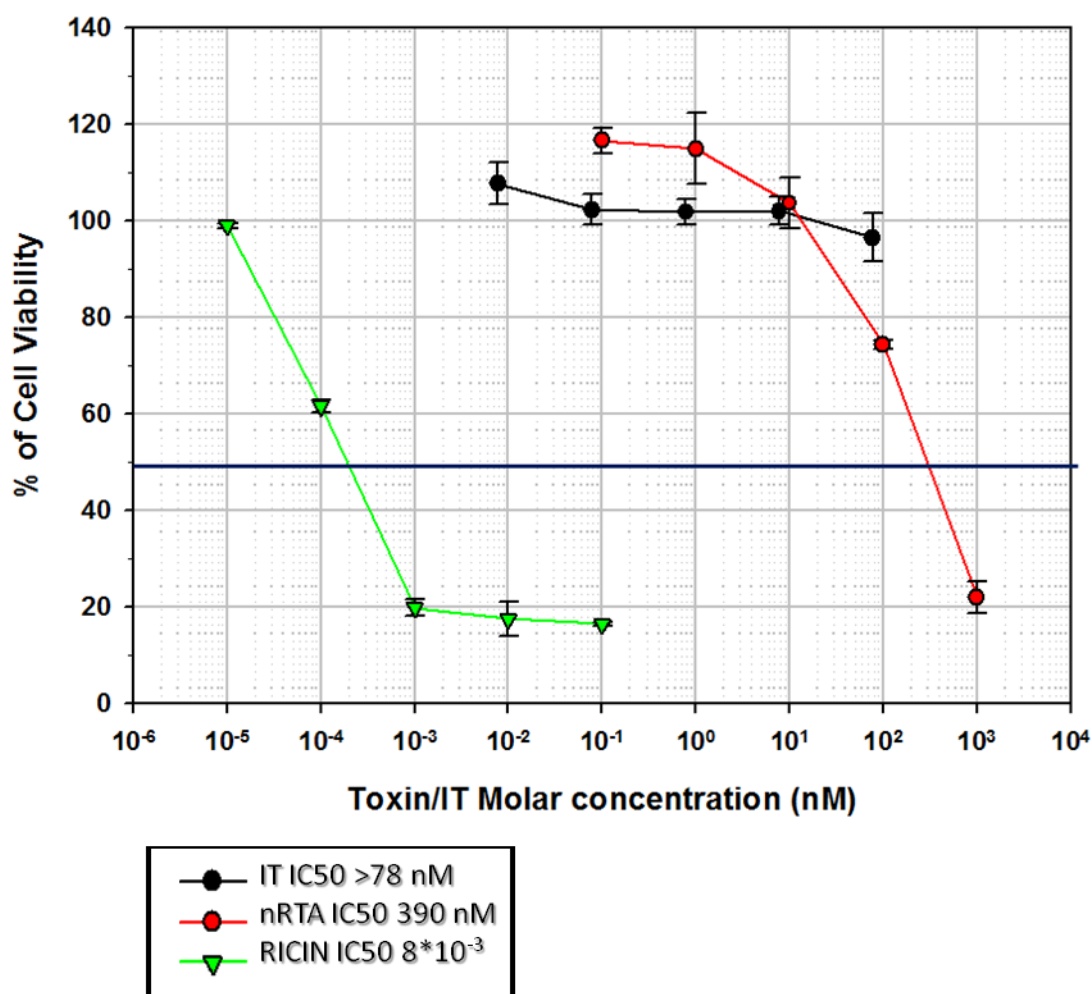


Fig. 24 Dose-response curves obtained treating HEK293 wt with increasing concentration of molecules described in the picture. The cytotoxic efficacy was measured considering the absorbance of water-soluble orange colored product after 36 hrs of incubation. IT – immunotoxin; nRTA anti-meso-nRTA; RICIN – ricina.

The same assay was carry out on other mesothelin negative cell lines A431, PC3, JURKAT and CEM. Table 16 showed that chemical IT does not have cytotoxic activity on mesothelin negative cells confirming an high specificity of binding and recognition of its target antigen expressed on the cell surface.

Results

XXT ASSAY 36 hrs

CELL LINES	IT (nM)	Toxin (nM)	RICIN (nM)
A431	> 78	> 1,000	0.01
PC3	> 78	> 1,000	0.003
JURKAT	> 78	150	n.d.
CEM	> 78	384	n.d.

Table 16 Dose-response data obtained treating mesothelin negative cells A431, JURKAT, PC3, CEM. The cytotoxic efficacy was measured considering the absorbance of water-soluble orange colored product after 36 hrs of incubation. IT- immunotoxin; TOXIN - anti-meso-nRTA; RICIN - ricina.

The cytotoxic activity was also investigated on positive (HEK293-mesothelin) and negative cell lines (HEK293 wt, A431, PC3) after 72 hrs of incubation with immunotoxin (IT), toxin (nRTA) and Ricin to detect if chemical IT increases the specific toxicity on a more long time of its permanence on cells. As illustrated in the table 17 chemical immunotoxin, maintained an high specific cytotoxic killing (0.09 nM) superimposable to that of 36 hrs of incubation.

XXT ASSAY 72 hrs

CELL LINES	IT (nM)	Toxin (nM)	RICIN (nM)
HEK293-meso	0.09	157	0.000015
HEK293 wt	> 78	70	0.000031
A431	> 78	580	n.d.
PC3	> 78	558	n.d.

Table 17 Dose-response data obtained treating mesothelin positive cells (HEK 293-mesothelin) and mesothelin negative cells (HEK293 wt, A431, PC3). The cytotoxic efficacy was measured considering the absorbance of water-soluble orange colored product after 72 hrs of incubation. IT- immunotoxin; TOXIN - anti-meso-nRTA; RICIN - ricina.

We can conclude that the conjugation of anti-mesothelin mAb to the toxins substantially potentiates the toxicity of RTA against mesothelin positive cells.

As a further proof of specificity, we also incubated, in a classic cytotoxic assay, HEK293-mesothelin cells with a fixed concentration of anti-mesothelin mAb–spdp-RTA IT (0,03 nM) in the presence of increasing molar excess of the unconjugated anti-

Results

mesothelin mAb (e.g. 10X, 100X, 1,000X), to displace chemical IT binding and specifically prevent cell intoxication. As illustrated in Figure 25 the cytotoxic effect of IT on HEK293-mesothelin cells was completely removed using a 1,000 molar excess of anti-mesothelin mAb; this observation supports the concept of an antibody-mediated and specific cytotoxicity of our IT.

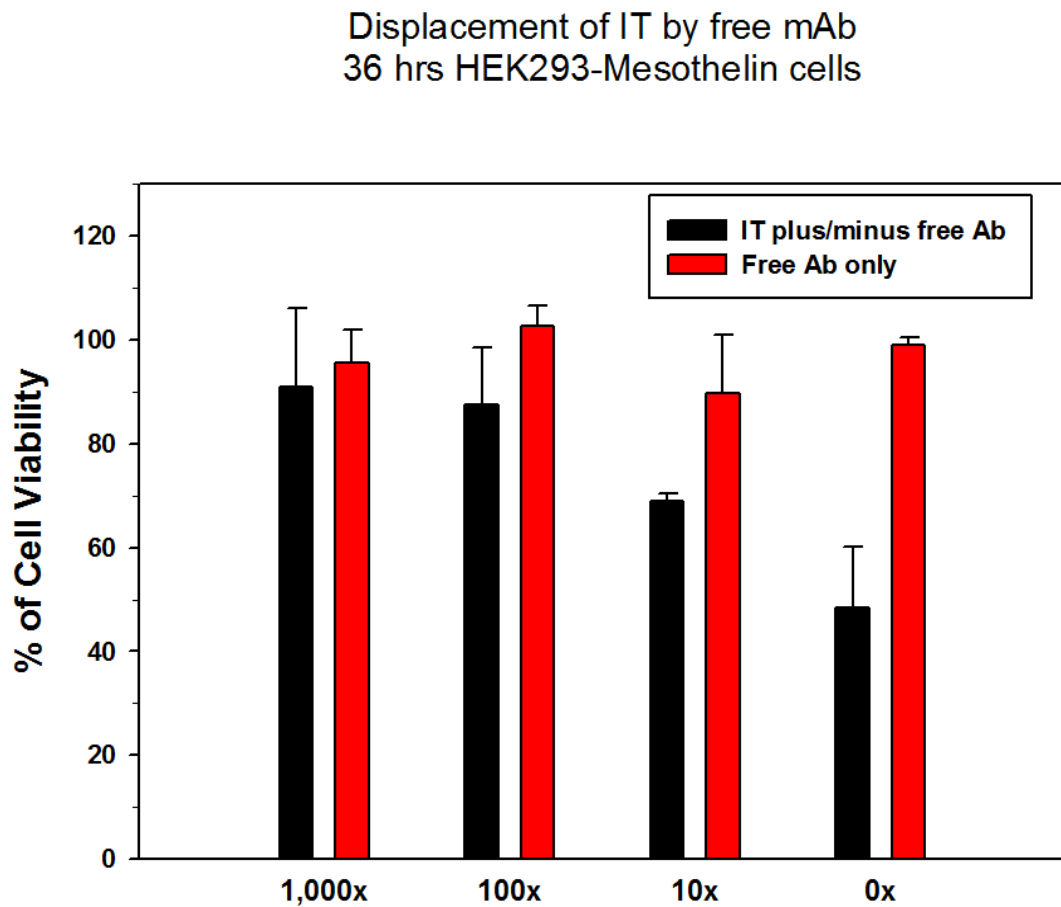


Fig. 25 The cytotoxic effect of the IT on HEK293-mesothelin cells was decreased using increasing concentration of anti-mesothelin mAb.

DISCUSSION

Cancer is the second leading cause of human death next to coronary disease. Worldwide, millions of people die from cancer every year and deaths generally are on the rise. Conventional therapies, like surgery, radiotherapy and chemotherapy, are not always sufficient to eliminate the tumor and the possible metastases; moreover these therapies are responsible of many side effects. For this reason there is an urgent need for new treatments strategies, the most promising one is probably immunotherapy.

In this context, the mesothelin is a membrane glycoprotein of about 40 kDa, whose expression at physiologic tissues is limited to the mesothelial cells of serosa (pleura, pericardium and peritoneum). The protein is instead overexpressed in different tumoral histotypes, in particular in ovarian, pancreatic and lung carcinoma so as in all mesothelioma. A restricted distribution of the mesothelin through physiologic tissues and, in the opposite, an overexpression in many neoplastic histotypes make that protein be a promising target for imaging procedures and for the specific tumor treatments. Moreover, in many patients affected with ovarian carcinoma or mesothelioma, the mesothelin is shedded in the serum, supposing a potential usage as a biomarker.

Nowadays, there are various studies aiming at finding antibodies that are specific for the mesothelin and some molecules have just entered in clinical trials: CAT-5001 (recombinant immunotoxin) and MORAb-009 (chimerical antibody). Since both molecules contain some murine portions, once given to patients they can determine the development of HAMA (Human Anti-Mouse Antibody) reactions; the necessity to create some full human Abs arises from that. In this contest, an article by the Ho M. and

Discussion

colleagues has recently (May 2011) been published, where a complete human antibody HN1 (scFv) directed to the mesothelin is presented (Ho M. *et al.*, 2011). This antibody acts by sequestering mesothelin and so by preventing its binding to CA125 protein on cancer cells. In addition, the HN1 IgG kills cancer cells with very strong antibody dependent cell mediated cytotoxicity (ADCC). Moreover, there is in the same study some information about the creation of an immunotoxin composed by HN1 fused with PE38 toxin (the truncated portion of the exotoxin A of *Pseudomonas Aeruginosa*). This immunotoxin proved to be able to selectively eliminate tumor cells overexpressing the mesothelin with high cytotoxic activity.

Because commercial mAb to mesothelin is not already available, due to patent restriction the aim of my thesis was to produce a murine monoclonal antibody specific for the mesothelin to eventual usage in diagnosis and therapy.

Once obtained the specific-mesothelin c-DNA has been amplified by PCR and inserted inside vectors for expression in prokaryote and eukaryote. Then, the cloned sequence functionality has been evaluated through the production, respectively, of the recombinant protein in bacteria, and through the creation of transfected cell lines. The recombinant protein has been shaped by transforming a bacteria strain of E. Coli, BL21 DE3 with the pET30a vector; then, the protein has been purified on a chromatographic column by affinity. Inclusion bodies have been previously lysed, while the protein had been refolded before purifying. In the course of the refolding the protein showed a high tendency to aggregate, especially in saline solutions; the remedy to that was developing a refolding protocols based on low salt concentration buffers. Moreover, the protein has been expressed on HEK293 cell membrane through transfection with pcDNA3.1-

Discussion

mesothelin vector. FACS analyses demonstrated that the transfection took place successfully (MFI = 8,650 for transfected cells e MFI = 216 for negative control).

The recombinant protein and the transfected cells overexpressing the antigen of interest were fundamental instruments for the execution of the project. In fact, they were useful reagents for the screening of the hybridomas obtained by the fusion after mice immunization, because we need simple, fast and relatively low cost assays to analyze the mAb specificity. In our case, the assays we decided to use were ELISA assays and flow cytometry.

Then, reagents were essential to perform the mouse immunization protocols; in fact, in our laboratory the best results have been obtained using cell membranes of cells overexpressing the antigen of interest during the first phases (see i.p. injections, material and methods). These stimulate the antigenic response and in the specific case of membrane proteins, they tend to maintain their native form also dimer, allowing the generation of Abs able to identify the target protein on cells and, therefore, to get Abs for applications in diagnostic and thereutical procedures. The recombinant protein, on the contrary, has been used for boosters (see i.v. injections, material and methods) before the fusion, due of its solubility.

Subsequently, polyclonal hybridomas have been obtained through Kohler and Milstein technique. Balb/c mice were immunized by injection of membranes of cell lines expressing the antigen in the native (OVCA-3) or transfected (HEK293-mesothelin) form, and of the recombinant protein produced in the bacteria.

Discussion

The flow cytometer analysis allowed us to check the presence of anti-mesothelin antibodies in the sera of immunized mice. The supernatants of 48 polyclonal hybridomas obtained after the fusion were tested by FACS to evaluate the presence of Abs specific for the mesothelin. The analysis was done on HEK293 wt cells and HEK293-mesothelin and brought to the identification of a unique out of 48 positive polyclonal hybridoma; in fact, the others showed a value of MFI on HEK293-mesothelin which was comparable with the value of MFI obtained with HEK293 wt cells. The same analyses was conducted by ELISA assay where all the hybridomas were positive; that result depended from an antibody response to the Ag recombinant that probably expressed more immune-dominant epitopes than the initial shape.

A prior fusion obtained after animal immunization with cell membranes (i.p. injected) and recombinant protein (i.v. and i.p. injected instead that only i.v.) generated only hybridomas that recognized the recombinant mesothelin (data not showed). In our case it is therefore very important the choice of the immunization protocol, because the antibody response developed against recombinant protein does not allow to obtain with a good frequency Abs that also recognize the proper folding of the protein.

The only positive polyclonal hybridoma has been analyzed in three flow cytometer assays at a interval of eight days and it proved that the hybridoma does not lose the ability to produce Abs specific. At this point, the specific polyclonal hybridoma has been cloned by the Limiting Dilution Analyses technique and the supernatants of the monoclonal hybridomas have been analyzed by FACS on HEK293 wt cells and HEK293-mesothelin cells. 34 clones resulted positive and two were chosen among them for further analyses: clones A and B. Clones A and B were further tested by FACS

Discussion

analysis on cells expressing the mesothelin in the native form (OVCAR-3) and on a panel of antigen negative cell lines: C6, U937, A431, PC3, Jurkat. This last analyses demonstrated that clones A and B produce specific antibodies against mesothelin and that those antibodies are able to recognize the antigen also on cell lines which express it constitutively. The lack of cross-reactions on the mesothelin-negative cell lines has been proved.

For additional characterization studies, we needed to produce high amounts of monoclonal antibodies of interest and purify them. So, it was necessary to adapt clones to the growth in a medium devoid of FCS: Hybridomed. The use of that medium allows ultra-pure preparations of the Ab of interest excluding possible contaminations by bovine immunoglobulin. The antibodies were purified on a chromatographic column by affinity and were quantified by absorbance evaluation at 280 nm using a spectrophotometer and by BCA assay. From about 250 ml of culture medium of each clone 3 mg of Ab-anti-mesothelin were obtained. The purified Ab was farther tested by flow cytometry to check that after purification it would have maintained the ability to specifically recognize mesothelin; the antibodies were eluted from the columns of affinity with low pH buffers, conditions that in some cases can inactivate their functionality.

At this point of the project it was interesting to verify if the antibodies produced by the two clones were identical; this could be partly proven, studying the capability of both biotinylated Ab of clones to displacement each other in the antigen binding (data not shown). To have a sure response we used the technology of recombinant DNA

Discussion

identifying the variable regions (VH and VL) of the two Abs through PCR and sequence.

Sequence brought to the conclusion that the two cell clones A and B produce the same Ab because they contain the same light and heavy variable regions, so they recognize the same epitope and with the same affinity.

Finally, the affinity of Ab for the mesothelin was evaluated by binding studies carried out on HEK-293-mesothelin cells. Scalar concentrations of Ab of interest and of commercially available mAb K1 were used for the assay. Then, the samples were incubated with the secondary Ab Goat Anti Mouse FITC at saturating concentrations and analyzed by flow cytometry. From that analysis, our Ab resulted to have a better affinity than the mAb K1; in fact, our Ab saturates the mesothelin molecules present on the membranes of the cells HEK293-mesothelin at a concentration of about 9 nM, while mAb K1 requires for slightly higher concentrations (18 nM).

Next step will consist on testing on a wider panel of positive cells our Ab to confirm that it is able to recognize the antigen expressed constitutively on different cell lines.

The good binding properties in its native form of our “home-made” anti-mesothelin mAb, represented a good prerequisite to synthesize immunoconjugates for either diagnostic and therapeutic applications. We here produced chemical ITs (anti-mesothelin mAb–spdp-nRTA) that showed powerful and specific cytotoxic efficacy against mesothelin-positive cells, even better than nRTA alone. The same chemical ITs did not show any cytotoxic effect against mesothelin-negative cells.

Discussion

In the cytotoxic experiments anti-mesothelin mAb–spdp-RTA chemical IT don't have toxicity on mesothelin negative cells (IC_{50} values don't detectable); moreover chemical IT is 6,433 and 1744 fold more toxic than the nRTA alone on HEK293-mesothelin cells after 36 hrs and 72 hrs of incubation respectively. This large “therapeutic windows” between mesothelin positive and negative cells represent for anti-mesothelin mAb-spdp-nRTA chemical IT a good preliminary proof of efficacy for further in vivo experiments using mouse models of malignant mesothelioma, pancreatic, ovarian and lung adenocarcinoma.

Abovementioned data are even more encouraging once considered that there are many further perspectives for future developments regarding the use of our “home-made” anti-mesothelin mAb.

First of all, the sequence identification of the variable regions (VH and VL) will allow us to proceed with the synthesis of scFv antibody fragments showing more reduced dimensions; so we could have mesothelin-specific tool and a molecule suitable for further genetic engineering. The obtained scFv will be tested on mesothelin-positive cells to prove its functionality and specificity. When compared to their whole molecule counterparts such smaller-size proteins have a lower plasma half life because scFvs are more rapidly cleared from the blood by renal filtration. On contrary these small proteins have better penetration rates in the tumor mass (known to have an high interstitial pressure) to improve the total killing of tumor cells. Paradoxically the lower affinity of single chain antibodies, in respect to the whole molecule counterpart, can itself lead to a better penetration into the core of tumor masses, overcoming the so called “antigen site barrier” that otherwise leads to an early and massive binding of highly affine antibodies

Discussion

to their cognate ligands in the outer layers of tumor mass, preventing a deeper penetration. All abovementioned properties lead to an improvement in tumor-blood ratio concentrations for scFv reagents. This property is particularly favourable for radioimmunoimaging/therapeutical applications once reagents are sequentially administered; pre-targeted single chain antibodies, either monomeric or multimeric with unique or different specificity, can be administered in high doses, in order to reach the highest concentration inside the tumor, with few side effects. In the following loading phase cytotoxic moiety can be rapidly and efficiently directed to the pretargeted site. This strategy leads to an improvement of the therapeutic index once compared to the administration of radioimmunoconjugates as single molecules, because of fewer side effects for the same dose of radioactivity.

We than will produce engineered fusion protein (scFv anti-mesothelin – PE40) and the powerful and specific cytotoxic action against mesothelin-positive cells will be measured. Fusion ITs are more stable, easier to produce in large amount and have highly reproducible chemical properties due to the constant 1:1 antibody:toxin ratio.

One possible problem of the IT is related to the immunogenicity of both scFv and toxic moiety due to the well-known HAMA and HATA (anti-toxin antibody) responses. Possible measures to overcome this problem will include humanization of scFv.

Another interesting field of application of our “home-made” anti-mesothelin antibodies is represented by antibody functionalized nanoparticles for tumor targeting (AFNaTT). Nanoparticles are in fact of great interest for use in biomedicine as imaging tools, phototherapy and heat therapy agents and also for gene carrying; due to their very low dimension, in the “mesoscopic” size range of about 10- to 100-nm diameter, these structures show some interesting characteristics: optimal surface areas for conjugation

Discussion

of carriers molecules such as antibody or their scFv fragment, a volume that allow their loading with high amounts of trackers agents (i.e. optical, radioisotopic or magnetic) but at the same time a dimension consistent to an efficient blood stream carriage and a substantial extravasation at the tumor site.

Gold NP are also very interesting for their chemical inertness; they are resistant to oxidative conditions which permits a direct linking of different organic/biological molecules via gold-sulphur bond or electrostatic attraction. Gold itself shows a biological inertness and no long-term toxicity, as proved by its historical use in different clinical setting from dental implantology to the management of the rheumathoid arthritis.

Gold NP shows optical properties that can be enhanced by linking at their surface SERS (surface enhanced Raman scattering) molecules; SERS amplifies the Raman scattering of about 10¹⁴- to 10¹⁵-fold, allowing detection and identification of an even limited number of nanoparticles. Optimized Raman signal has to date increasing applications in tumor imaging, taking advantage of some peculiar physical properties. Raman peaks are in fact narrower in respect to conventional fluorescence ones, allowing an easier signal detection in complex systems such as tissues having many potentially interferent autofluorescence signals (i.e. heme groups, flavonoids etc.).

Finally, there are many further perspectives for future developments regarding the use of our anti-mesothelin mAb as its biotechnological derivates. By actually our on-going collaborations in the field of cancer diagnosis and therapy we will can:

_ to use T cell chimeric receptors containing anti-mesothelin scFv as their extracellular recognition elements will can redirect the specificity of T cells in an MHC-independent manner (T-body);

Discussion

_ to evaluate the apoptotic effect of fusion protein scFv anti-mesothelin-TNF α on mesothelin positive tumor cells;

_ to evaluate the ability of scFv anti-mesothelin-DOTA-Radionuclide conjugate in tumor imaging and therapeutical applications. The goal of this project could be to produce in GMP conditions Ab and scFv and to start with phase 0 clinical trials after a preclinical setting in mouse models;

_ to develop bispecific scFv, anti-mesothelin and anti-CD16, which are able to redirect NK cells at the tumor site. It is known that NK cells are less prone to be influenced by immunosuppressive tumor environment;

_ to evaluate the efficacy of pre-targeting methods based on bispecific Ab anti-mesothelin and anti-hapten, created using the Dock and Lock method.

After a “pre-loading” of specific antibody at tumor site (via venous systemic injection), a radiolabeled hapten will be injected in order to be captured, in the same site, from the “resident” specific Ab.

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